National Toxicology Program
U.S. Department of Health and Human Services

Center for the Evaluation of Risks to Human Reproduction

NTP-CERHR MONOGRAPH ON SOY INFANT FORMULA

September 2010 *NIH Publication No. 10-5995*

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PREFACE

Soy infant formula contains soy protein isolates and is fed to infants as a supplement to or replacement for human milk or cow milk. Soy protein isolates contains estrogenic isoflavones ("phytoestrogens") that occur naturally in some legumes, especially soybeans. Phytoestrogens are non-steroidal, estrogenic compounds. In plants, nearly all phytoestrogens are bound to sugar molecules and these phytoestrogen-sugar complexes are not generally considered hormonally active. Phytoestrogens are found in many food products in addition to soy infant formula, especially soy-based foods such as tofu, soy milk, and in some over-the-counter dietary supplements. Soy infant formula was selected for NTP evaluation because of:

(1) the availability of large number of developmental toxicity studies in laboratory animals exposed to the isoflavones found in soy infant formula (namely, genistein) or other soy products, as well as a number of studies on human infants fed soy infant formula,
(2) the availability of information on exposures in infants fed soy infant formula, and
(3) public concern for effects on infant or child development.

On October 2, 2008 (73 FR 57360), the National Toxicology Program (NTP) Center for the Evaluation of Risks to Human Reproduction (CERHR) announced its intention to conduct an updated review of soy infant formula in order to complete a previous evaluation that occurred in 2006. Both the current and previous evaluations relied on expert panels to assist the NTP in developing its conclusions on the potential developmental effects associated with use of soy infant formula, presented in the NTP Brief on Soy Infant Formula. The initial expert panel met on March 15 – 17, 2006 to reach conclusions on the potential developmental and reproductive toxicities of soy infant formula and its predominant isoflavone constituent genistein. The expert panel reports were released for public comment on May 5, 2006 (71 FR 28368). On November 8, 2006 (71 FR 65537), CERHR staff released draft NTP Briefs on Genistein and Soy Formula that provided the NTP's interpretation of the potential for genistein and soy infant formula to cause adverse reproductive and/or developmental effects in exposed humans. However, CERHR did not complete these evaluations, finalize the briefs, or issue NTP Monographs on these substances based on this initial evaluation.

Since 2006, a substantial number of new publications related to human exposure or reproductive and/or developmental toxicity have been published for these substances. Thus, CERHR determined that updated evaluations of genistein and soy infant formula were needed. However, the current evaluation focuses only on soy infant formula and the potential developmental toxicity of its major isoflavone components, e.g., genistein, daidzein (and estrogenic metabolite, equol), and glycitein. This updated evaluation does not include an assessment on the potential reproductive toxicity of genistein following exposures during adulthood as was done in the 2006 evaluation. CERHR narrowed the scope of the evaluation because the assessment of reproductive effects of genistein following exposure to adults was not considered relevant to the consideration of soy infant formula use in infants during the 2006 evaluation. To obtain updated information about soy infant formula for the CERHR evaluation, the PubMed (Medline) database was searched from February 2006 to August 2009 with genistein/genistin, daidzein/daidzin, glycitein/glycitin, equol, soy, and other relevant keywords. References were also identified from the bibliographies of published literature.

The updated expert panel report represents the efforts of a 14-member panel of government and non-government scientists, and was prepared with assistance from NTP staff. The finalized report,

released on January 15, 2010 (75 FR 2545), reflects consideration of public comments received on a draft report that was released on October 19, 2009 for public comment and discussions that occurred at a public meeting of the expert panel held December 16–18, 2009 (74 FR 53509).

The finalized report presents conclusions on the:

(1) strength of scientific evidence that soy infant formula or its isoflavone constituents are developmental toxicants based on data from *in vitro*, animal, or human studies;
(2) extent of exposures in infants fed soy infant formula;
(3) assessment of the scientific evidence that adverse developmental health effects may be associated with such exposures; and
(4) knowledge gaps that will help establish research and testing priorities to reduce uncertainties and increase confidence in future evaluations.

The Expert Panel expressed *minimal* concern for adverse developmental effects in infants fed soy infant formula. This level of concern represents a "2" on the five-level scale of concern used by the NTP that ranges from *negligible* concern ("1") to *serious* concern ("5").

The Expert Panel report on Soy Infant Formula was considered extensively by NTP staff in preparing the 2010 NTP Brief on Soy Infant Formula, which represents the NTP's opinion on the potential for exposure to soy infant formula to cause adverse developmental effects in humans. The NTP concurred with the expert panel that there is *minimal concern* for adverse effects on development in infants who consume soy infant formula. This conclusion was based on information about soy infant formula provided in the expert panel report, public comments received during the course of the expert panel evaluation, additional scientific information made available since the expert panel meeting, and peer reviewer critiques of the draft NTP Brief by the NTP Board of Scientific Counselors on May 10, 2011.[2] The Board voted in favor of the minimal concern conclusion with 7 yes votes, 3 no votes, and 0 abstentions. One member thought the conclusion should be *negligible* concern and 2 members thought the level of concern should be higher than *minimal* concern. The NTP's response to the May 10, 2010 review ("peer-review report") is available on the NTP website at *http://ntp.niehs.nih.gov/go/9741*. This monograph includes the NTP Brief on Soy Infant Formula as well as the final Expert Panel report on Soy Infant Formula. An abbreviated version of the final Expert Panel report was published in Birth Defects Research Part B **[Volume 92, Issue 5, October 2011, Pages: 421–468]**. Public comments received as part of the NTP's evaluation of soy infant formula and other background materials are available at *http://cerhr.niehs.nih.gov/evals/index.html.*

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[2] Meeting materials are available at *http://ntp.niehs.nih.gov/go/9741*.

ABSTRACT

NTP MONOGRAPH ON THE POTENTIAL HUMAN REPRODUCTIVE AND DEVELOPMENTAL EFFECTS OF SOY INFANT FORMULA

Soy infant formula contains soy protein isolates and is fed to infants as a supplement to or replacement for human milk or cow milk. Soy protein isolates contains estrogenic isoflavones ("phytoestrogens") that occur naturally in some legumes, especially soybeans. Phytoestrogens are non-steroidal, estrogenic compounds. In plants, nearly all phytoestrogens are bound to sugar molecules and these phytoestrogen-sugar complexes are not generally considered hormonally active. Phytoestrogens are found in many food products in addition to soy infant formula, especially soy-based foods such as tofu, soy milk, and in some over-the-counter dietary supplements.

Soy infant formula was selected for evaluation by the National Toxicology Program (NTP) because of the:

(1) availability of large number of developmental toxicity studies in laboratory animals exposed to the isoflavones found in soy infant formula (namely, genistein) or other soy products, as well as a number of studies on human infants fed soy infant formula,
(2) the availability of information on exposures in infants fed soy infant formula, and
(3) public concern for effects on infant or child development.

The NTP evaluation was conducted through its Center for the Evaluation of Risks to Human Reproduction (CERHR) and completed in September 2010.

The results of this soy infant formula evaluation are published in an NTP Monograph. This document contains the NTP Brief on Soy Infant Formula, which presents NTP's opinion on the potential for exposure to soy infant formula to cause adverse developmental effects in humans. The NTP Monograph also contains an expert panel report prepared to assist the NTP in reaching conclusions on soy infant formula. The NTP concluded there is *minimal concern* for adverse effects on development in infants who consume soy infant formula. This level of concern represents a "2" on the five-level scale of concern used by the NTP that ranges from *negligible* concern ("1") to *serious* concern ("5").

This conclusion was based on information about soy infant formula provided in the expert panel report, public comments received during the course of the evaluation, additional scientific information made available since the expert panel meeting in December 2009, and peer reviewer critiques of the draft NTP Brief by the NTP Board of Scientific Counselors on May 10, 2010 (*http://ntp.niehs.nih.gov/go/9741*).

National Toxicology Program
U.S. Department of Health and Human Services

Center for the Evaluation of Risks to Human Reproduction

NTP-CERHR BRIEF ON SOY INFANT FORMULA

September 16, 2010

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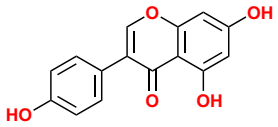
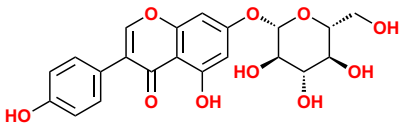
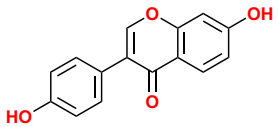
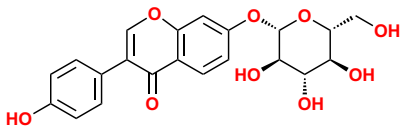
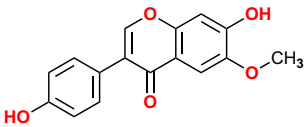
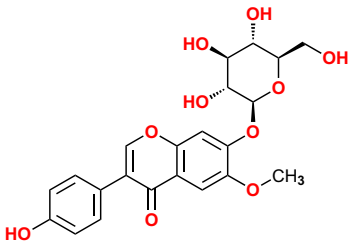
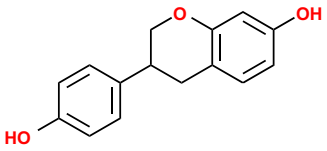
WHAT IS SOY INFANT FORMULA?

Soy infant formula is fed to infants as a supplement to or a replacement for human milk, or as an alternative to cow milk formula. In the United States, the Food and Drug Administration (FDA) regulates the nutrient composition of soy infant formula as well as other infant formula types such as cow milk formula. Infant formulas must comply with the Infant Formula Act of 1980 and subsequent amendments passed in 1986 (FDA 2000). The specified nutrient levels are based on the recommendations of the Committee on Nutrition of the American Academy of Pediatrics and are reviewed periodically as new information becomes available. In the United States, a relatively small number of companies market soy infant formula (see Expert Panel Report, Table 4). The primary ingredients in soy infant formula include corn syrup, soy protein isolate, vegetable oils, sugar, vitamins, minerals, and other nutrients. Soy protein isolate is made from soybeans and is present in infant formulas at 14–16% by weight. In addition, the formulas are fortified with nutrients such as iron, calcium, phosphorous, magnesium, zinc, manganese, copper, iodine, sodium selenate, potassium, chloride, choline, inositol, and vitamins A, C, D, E, K, and B (B1, B2, B6, B12, niacin, folic acid, pantothenic acid, and biotin). Contaminants of soy protein include phytates (1.5%), which bind minerals and niacin, and protease inhibitors, which have antitrypsin, antichymotrypsin, and antielastin properties. Formulas are fortified with minerals to compensate for phytate binding and heated to inactivate protease inhibitors. Aluminum from mineral salts is found in soy infant formulas at concentrations of 600–1300 ng/mL, levels that exceed aluminum concentrations in human milk, 4–65 ng/mL (Bhatia and Greer 2008). The typical reconstitution of powdered formula is the addition of 8.7–9.3 g powdered formula to 2 fluid ounces of water (Drugstore.com 2004). Soy infant formulas are also available as concentrated liquids (generally 1 part soy infant concentrate to 2 parts water) and as ready-to-feed formulations.

Soy protein isolate contains isoflavones with estrogenic activity called "phytoestrogens," a subset of plant-derived compounds with biological activity similar to the female hormone estrogen that occurs naturally in some legumes. Phytoestrogens are found in many soy-based food products in addition to soy infant formula, such as tofu and soy milk, and in some over-the-counter dietary supplements. In soy infant formula, nearly all the phytoestrogens are bound to sugar molecules and these phytoestrogen-sugar complexes ("glucosides") are not generally considered hormonally active. There are three major glucosides found in soy infant formula: genistin, daidzin, and glycitin (**Figure 1**). Before isoflavone glucosides can be absorbed into the systemic circulation, they are typically first hydrolyzed to their sugar-free forms ("aglycones"). In addition, several studies show that isoflavones can also be absorbed as glucosides (Allred *et al.* 2005; Hosoda *et al.* 2008; Kwon *et al.* 2007; Steensma *et al.* 2006). The sugar-free forms of these phytoestrogens are the biologically active forms and are called genistein, daidzein, and glycitein, respectively. In some people, daidzein also produces an estrogenic metabolite called equol. Glycosidase activity occurs in food products (naturally by endogenous enzymes or those added during processing), in the cells of the gastrointestinal mucosa, or in colon microbes, and isoflavones can be measured in blood within an hour of soy ingestion (Kano *et al.* 2006; Larkin *et al.* 2008). Aglycones undergo passive diffusion across the small and large intestinal brush border (Larkin *et al.* 2008). Once absorbed, the body then binds, i.e. conjugates, the free phytoestrogens to another molecule such as glucuronic acid. As much as 97–99% of the phytoestrogens in human blood are bound, or conjugated, to another molecule. The relative amounts of phytoestrogens in soy infant formula are genistin > daidzin > glycitin, which also corresponds to their relative estrogenic potency based on *in vitro* estrogen-receptor activities of the sugar-free formstheir relative estrogenic potency based on *in vitro* estrogen-receptor activities of the sugar-free forms of these phytoestrogens (UK Committee on Toxicity 2003).

Figure 1. Chemical Structures of Isoflavones Associated with Soy Formula

Isoflavone	*Structure*	*Isoflavone*	*Structure*
Genistein $C_{15}H_{10}O_5$ MW: 270.24 CAS RN: 446–72–0		**Genistin** $C_{21}H_{20}O_{10}$ MW: 432.37 CAS RN: 529–59–9	
Daidzein $C_{15}H_{10}O_4$ MW: 254.24 CAS RN: 486–66–8		**Daidzin** $C_{21}H_{20}O_9$ MW: 416.37 CAS RN: 552–66–9	
Glycitein $C_{16}H_{12}O_5$ MW: 284.26 CAS RN: 40957–83–3		**Glycitin** $C_{22}H_{22}O_{10}$ MW: 446.41 CAS RN: 40246–10–4	
Equol $C_{15}H_{14}O_3$ MW: 242.27 CAS RN: 531–95–3			

USE OF SOY INFANT FORMULA AND EXPOSURE TO ISOFLAVONES IN INFANTS AND ADULTS

USAGE

Sales of soy infant formula represented ~13% of the United States infant formula market based on 2009 dollar sales (personal communication with Robert Rankin, Manager of Regulatory and Technical Affairs at the International Formula Council, October 13, 2009). The use of soy infant formula in the United States has decreased by almost half between 1999 and 2009, from 22.5% to 12.7%, calculated based on total formula sold corrected for differences in formula cost.[2] The usage and sales of soy infant formula vary worldwide, ranging from 2 to 7% of infant formula sales in the United Kingdom, Italy, and France, and 13% in New Zealand (Agostoni *et al.* 2006; Turck 2007), to 31.5% in Israel (Berger-Achituv *et al.* 2005).

Recent data from the Infant Feeding Practices Study II (IFPS II), a longitudinal mail survey of mothers of infants conducted by the FDA in 2005–2007, indicated that ~57 to 71% of infants were fed infant formula (of any kind) during the first 10 months of life (Grummer-Strawn *et al.* 2008). However, many aspects of infant formula use from this study are unknown, including what percent of infants were exclusively fed infant formula compared to what percent were fed a mixture of infant formula and breast milk. It is also unknown what proportion of formula-fed infants were exclusively fed soy infant formula, although it is not likely a large percentage. For example, in one prospective cohort study where parents chose the feeding method, only 23% of infants included in the "soy infant formula" group were exclusively fed soy infant formula from birth to 4 months of age (Gilchrist *et al.* 2009). In a study of Israeli infants (3–24

[2] Public comment from the International Formula Council (IFC), received December 3, 2009 (available at *http://cerhr.niehs.nih.gov/chemicals/genistein-soy/SoyFormulaUpdt/SoyFormula-mtg.html*) and personal communication with Dr. Haley Curtis Stevens, IFC.

months old), only 21.4, 16, and 18.5% of infants included in the "soy" group were exclusively fed soy infant formula the first year of life, the second year of life, or the first two years of life, respectively (Zung *et al.* 2008). Another study of feeding patterns in Israeli infants reported that of the formula-fed infants, 9% were started with a soy infant formula, but 50% were switched to a cow milk-based formula at some time (Nevo *et al.* 2007). This study also found that the type of formula used was changed for 47% of the formula-fed infants during the first 6 months of life, and that 12% had more than two changes.

Commonly cited reasons for use of soy infant formula are to feed infants who are allergic to dairy products or are intolerant of lactose, galactose, or cow-milk protein (Essex 1996; Tuohy 2003). In May 2008, the American Academy of Pediatrics (AAP) released an updated policy statement on the use of soy protein-based formulas (Bhatia and Greer 2008). The overall conclusion of the AAP was that although isolated soy protein-based formulas may be used to provide nutrition for normal growth and development in term infants, there are very limited indications for their use in place of cow milk-based formula. The only circumstances under which the AAP recommends the use of soy infant formula are instances where the family prefers a vegetarian diet or for the management of infants with galactosemia or primary lactase deficiency (rare). Soy infant formula is not currently recommended for preterm infants by the AAP or the European Society for Paediatric Gastroenterology, Hepatology and Nutrition (ESPGHAN) Committee on Nutrition (Agostoni *et al.* 2006).

Specific conclusions in the 2008 AAP report are:

- Lactose free and reduced lactose-containing cow milk formulas are now available and could be used for circumstances in which elimination or a reduction in lactose in the diet, respectively, is required. Because primary or congenital lactase deficiency is rare, very few individuals would require a total restriction of lactose. Lactose intolerance is more likely to be dose dependent. Thus, the use of soy protein-based lactose-free formulas for this indication should be restricted.

- The routine use of isolated soy protein-based formula has no proven value in the prevention or management of infantile colic or fussiness.

- Isolated soy protein-based formula has no advantage over cow milk protein-based formula as a supplement for the breastfed infant, unless the infant has one of the indications noted above.

- Soy protein-based formulas are not designed for or recommended for preterm infants. Serum phosphorus concentrations are lower, and alkaline phosphatase concentrations are higher in preterm infants fed soy protein-based formula compared to preterm infants fed cow milk-based formula. As anticipated from these observations, the degree of osteopenia is increased in infants with low birth weight receiving soy protein-based formulas. The cow milk protein-based formulas designed for preterm infants are clearly superior to soy protein-based formula for preterm infants.

- For infants with documented cow milk protein allergy, extensively hydrolyzed protein formula should be considered, because 10% to 14% of these infants will also have a soy protein allergy.

- Infants with documented cow milk protein-induced enteropathy or enterocolitis frequently are as sensitive to soy protein and should not be given isolated soy protein-based formula. They should be provided formula derived from hydrolyzed protein or synthetic amino acids.

- The routine use of isolated soy protein-based formula has no proven value in the prevention of atopic disease [i.e., hypersensitivity reactions, allergic hypersensitivity affecting parts of the body not in direct contact with the allergen] in healthy or high-risk infants.

Additional Sources of Soy Intake by Infants

A number of studies have reported on the use of soy foods in the context of infant feeding and feeding transitions during the first years of life.[3] Data from IFPS II indicated that ~6% of infants consume soy foods by 1 year of age (Grummer-Strawn *et al.* 2008). A survey of the isoflavone content of infant cereals in New Zealand led the authors to conclude that supplementation of the diet of a 4-month old infant fed soy infant formula with a single serving of cereal can increase isoflavone intake by more than 25%, depending on the brand used (Irvine *et al.* 1998). Infants may also be exposed to soy flour and soy oil by the use of soy-containing fortified spreads as a complementary food to address growth and nutritional issues in countries with high incidence of childhood malnutrition, such as Malawi (Lin *et al.* 2008; Phuka *et al.* 2008).

The consumption of soy milk by children is currently being assessed in the 2008 Feeding Infants and Toddlers Study (FITS), a survey of the eating habits and nutrient intakes of > 3,000 children from 4 to 24 months of age[4] sponsored by Nestle Nutrition Institute. Based on survey data collected in 2002, soy milk was reported as one of the more frequently consumed beverages in children 15–18 months of age, but not in younger infants or older toddlers 19–24 months of age (Skinner *et al.* 2004). A 2006 presentation from the Executive Director of the Soyfoods Association of North America, Nancy Chapman[5], cited 2002 FITS data to report that out of 600 toddlers surveyed, almost 4% consumed soy milk at least once a day. Overall, soy milk is one of the fastest growing markets in the soy food industry (United Soybean Board 2009). However, it is unclear whether this growth trend extends to infants and toddlers.

DAILY INTAKE AND BIOLOGICAL-BASED INDICATORS OF EXPOSURE

A number of studies in the United States and abroad have measured total isoflavone levels in infant formulas (see Expert Panel Report, Table 9). For infant formulas manufactured in the United States, the range of total isoflavone levels reported in reconstituted or "ready-to-feed" formulas was 20.9–47 mg/L formula (Franke *et al.* 1998; Setchell *et al.* 1998).[6] The range of total isoflavones content in soy infant formula samples collected in the United States and other countries is 10–47 mg/L (Genovese and Lajolo 2002; Setchell *et al.* 1998). Genistein is the predominant isoflavone found in soy infant formula (~58–67%), followed by daidzein (~29–34%) and glycitein (~5–8%). The isoflavone content in soy infant formula appears to be much less variable than the isoflavone content of soy beans or other soy products (e.g. soy supplements or soy protein isolates) (see Expert Panel Report, Section 1.2.2.4).

[3] Isoflavone exposure from these food items were not considered in the NTP evaluation of soy infant formula.

[4] Preliminary findings from the 2008 FITS are available at *http://medical.gerber.com/starthealthystayhealthy/FITSStudy.aspx*. The 2008 survey was sponsored by Nestlé Nutrition and conducted by Mathematica as a followup to the FITS 2002 study.

[5] Presentation available at *http://www.soyfoods.org/wp/wpcontent/uploads/2006/12/soymilk_in_school_meals.pdf*.

[6] The soy infant formula content of genistein (12.1–~31.2 mg/L or 44.4–~115.5 µM) (Franke *et al.* 1998; Setchell *et al.* 1998) is approximately 2.7×106 to 7.0×106 times higher than the maximum level of estradiol reported in frozen breast milk by Hines *et al.* (2007). In the Hines *et al.* (2007) study, estradiol was not detected in most samples and the maximum level detected was 4.5 pg/mL (0.000017 µM) from a frozen milk sample. The concentrations of estradiol in human milk reported in Hines *et al.* (2007) are lower than those reported in whole milk from Holstein cows (mean concentration = 1.4 pg/ml, range = <LOD to 22.9 pg/ml) (Pape-Zambito *et al.* 2007).

Table 1. Comparison of Estimated Intake of Genistein and Total Isoflavones in Infants Fed Soy Infant Formula to Other Populations

Population	Diet	Daily Intake, mg/kg bw/day*		Reference
		Total Isoflavone	Genistein	
Infants				
United States	Soy infant formula	2.3–9.3	1.3–6.2	Table 26 of Expert Panel Report
	Cow milk formula	0.0002–0.0158		Knight *et al.* 1998 Kuhnle *et al.* 2008
	Breast milk	0.0002–0.0063		Friar and Walker 1998 Setchell *et al.* 1998
Adults*				
United States	Omnivore	0.0097[a]–0.096[b]	0.005[a]–0.056[b]	Haytowitz 2009 [a] Tseng *et al.* 2008[b]
	Vegetarian	0.21	0.14	Kirk *et al.* 1999
European	Omnivore	0.007–0.009	0.004–0.005	Mulligan *et al.* 2007
	Vegetarian	0.100–0.112	0.057–0.062	
United Kingdom	Vegan	1.07	–	Friar and Walker 1998
Japanese	Traditional diet	0.67[b]	0.077[a]–0.43[b]	Fukutake *et al.* 1996[a] Arai *et al.* 2000[b]

*Daily intakes for adults were based on mg/day estimates presented in Table 25 of the Expert Panel divided by 70 kg body weight.

Infants fed soy infant formula have higher daily intakes of genistein and other isoflavones than other populations (Table 1). However, differences in methods used to select representative samples and calculate intake estimates limit the ability to compare intake estimates across studies, especially for dietary surveys. In addition, isoflavone intake appears to be highly variable in soy-consuming adult populations. Recognizing these caveats, the relative ranking of total isoflavone intake appears to be infants exclusively fed soy infant formula > vegan adults > Japanese adults consuming a traditional diet > vegetarian adults > omnivores consuming Western diets.

Infants fed soy infant formula also have higher blood-based levels of genistein and daidzein compared to other populations such as vegans and Asian populations consuming a traditional diet high in soy foods (Table 2). The latest findings for the United States, reported by Cao *et al.* (2009), were that concentrations of total genistein in whole blood samples from infants fed soy infant formula were 1455 ng/ml at the 75th percentile and 2763.8 ng/ml at the 95th percentile (personal communication with Dr. Yang Cao, NIEHS); both of these values are higher than the maximum total genistein concentrations available for any other population. The geometric mean of total genistein measured in these infants was 757 ng/ml, a value that is 53.3- and 70.1- times higher than the corresponding levels detected in infants fed cow milk formula or breast milk, respectively (Table 2). Average blood levels of total genistein in the soy infant formula-fed infants were ~160-times higher than the mean levels of total genistein in omnivorous adults in the United States (4.7 ng/ml) reported by Valentin-Blasini (2003); a similar pattern was observed for urinary concentrations of genistein and daidzein (Cao *et al.* 2009; U.S. Centers for Disease Control and Prevention 2008). It is not known for infants how long it takes to

Table 2. Average Blood-Based Levels of Genistein and Daidzein in Infants and Adult Populations

Population	Diet	Sample	Average Total Isoflavone Concentration, ng/ml (range)		Reference
			Genistein	Daidzein	
United States infants	Soy infant formula[a]	Whole blood	757 1455, 75th percentile	256 519, 75th percentile	Cao *et al.* 2009
		Plasma	684	295	Setchell *et al.* 1997
	Cow milk formula	Whole blood	14.2	5.5	Cao *et al.* 2009
		Plasma	3.16	2.06	Setchell *et al.* 1997
	Breastfed	Whole blood	10.8	5.3	Cao *et al.* 2009
		Plasma	2.77	1.49	Setchell *et al.* 1997
United States adults	Omnivores	Serum	4.7 (<LOD–203)	3.9 (<LOD–162)	Valentin-Blasini *et al.* 2003
Japanese men	Traditional diet	Plasma	105.2 (24–325)	71.3 (14.8–234.9)	Adlercreutz *et al.* 1994
Finnish women	Vegetarians	Plasma	4.6	4.7	
United Kingdom adults	Vegans/ Vegetarians	Plasma	40	20	Peeters *et al.* 2007

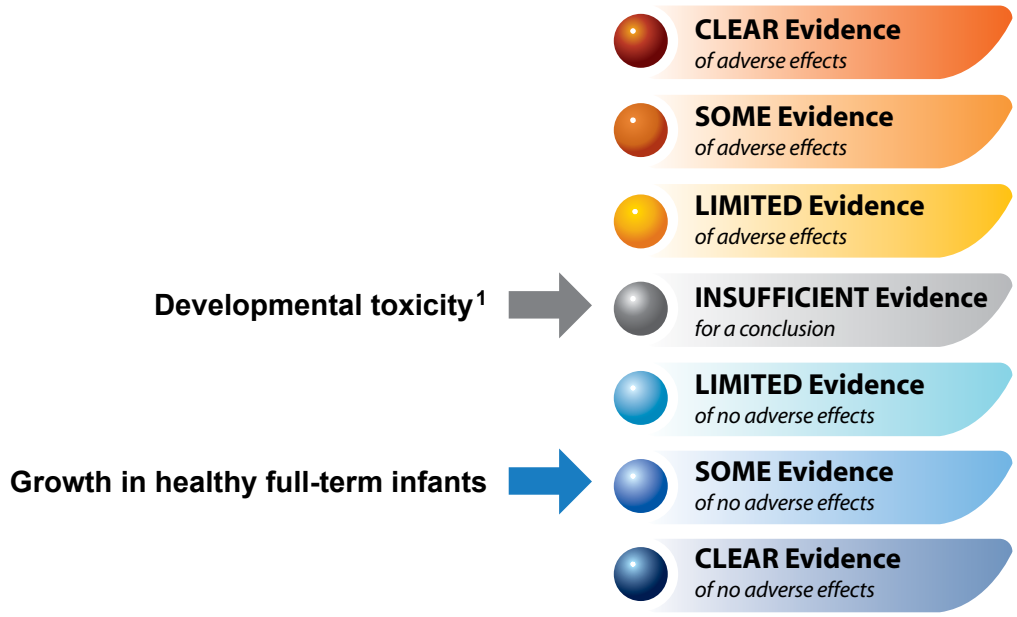
[a]Shaded cells are studies of infants fed soy infant formula.

achieve maximum blood concentrations of genistein and daidzein. In adults, length of time necessary to achieve maximum blood concentrations is ~5.7 and 6.2 hours, respectively (Cassidy *et al.* 2006), thus the blood levels of isoflavones sampled at least one hour after feeding as reported in Cao *et al.* (2009) may not represent the maximum concentration for each infant.

CAN SOY INFANT FORMULA OR ITS ISOFLAVONE CONTENTS ADVERSELY AFFECT HUMAN DEVELOPMENT?

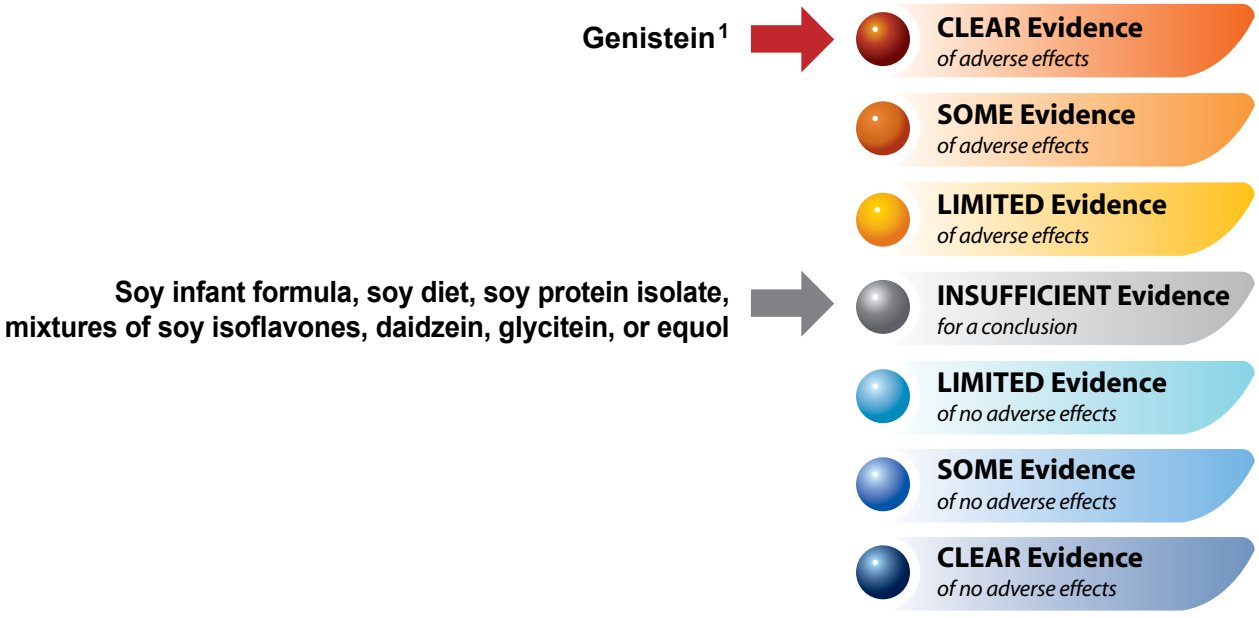
Appropriate levels of sex hormones are essential for normal development and function of the reproductive system. Because soy infant formula contains isoflavones with estrogen-like activity, concern has been expressed that feeding soy infant formula might adversely affect development of the reproductive system. There are presently not enough data from studies in humans to confirm or refute this possibility (**Figure 2**). Likewise, data from the studies in laboratory rodents and primates are not sufficient to permit a firm conclusion regarding the developmental toxicity of soy infant formula (**Figure 3**). However, blood levels of total genistein in infants fed soy infant formula can exceed blood levels in rats administered genistein in the diet or in mice treated by subcutaneous (sc) injection at dose levels that induce adverse developmental effects. Because of the high blood levels of isoflavones in infants fed soy infant formula and the lack of robust studies on the human health effects of soy

Figure 2. The Weight of Evidence that Soy Infant Formula or its Isoflavone Contents Causes Adverse Developmental Effects in Humans



[1] Based on consideration of the following endpoints: bone mineral density, allergy/immunology, thyroid function, reproductive endpoints, cholesterol, diabetes mellitus, and cognitive function.

Figure 3. The Weight of Evidence that Soy Infant Formula, Other Soy Products, or Individual Isoflavones Cause Adverse Developmental Effects in Laboratory Animals



[1] Manifested as: decreased age at vaginal opening; abnormal estrous cyclicity; decreased fertility, implants, and litter size; and histopathology of the female reproductive tract.

infant formula, the possibility that soy infant formula may adversely affect human development cannot be dismissed.

SUPPORTING EVIDENCE

Human Studies

There is a relatively large literature describing growth or other health parameters in infants fed soy infant formula. These studies provide sufficient evidence to conclude that use of soy infant formula does not impair growth during infancy in healthy full-term infants. However, this literature is considered insufficient to reach a conclusion on whether the use of soy infant formula adversely affects human development with respect to effects on bone mineral density, allergy/immunology, thyroid function, reproductive system endpoints, cholesterol, diabetes mellitus, and cognitive function (**Figure 2**). Commonly encountered limitations of these studies include: inadequate sample size, short-duration of follow-up, unspecified method of assignment to feeding groups, the use of self-selected breast- and formula-feeding mothers, changes in feeding methods (i.e., formula-type and/or breast milk), lack of information regarding the introduction of solid foods, and inadequate consideration of potential confounding variables. When the expert panel reviewed this literature, only 28 of the ~80 published human studies on soy infant formula were considered to have utility for the NTP-CERHR evaluation process (see **Expert Panel Report, Table 153**).

A number of critical research needs were also identified during the course of the evaluation based on case reports, pilot studies in humans, or findings in laboratory animals. In particular, there is a need to (1) assess the potential impacts of soy infant formula use on reproductive tissues or function during infancy, childhood, and later in life and (2) monitor soy infant formula fed-infants who have congenital hypothyroidism for possible decreases in the effectiveness of thyroid hormone replacement therapy, i.e., L-thyroxin. A discussion of the findings, conclusions, and research recommendations regarding effects of soy infant formula on growth and the gastrointestinal system, reproductive system and breast tissue, and thyroid function are described below.

Growth and Gastrointestinal Effects

Although the NTP considered the human studies insufficient to assess whether the use of soy infant formula adversely affects development, the NTP concurs with the expert panel that there is sufficient evidence to conclude that use of soy infant formula does not negatively impact growth in healthy, full-term infants. Of the 28 human studies considered by the expert panel to have utility for the NTP-CERHR, 13 of the studies assessed growth outcomes and 11 of 13 studies reported no decreases in growth measurements (Chan *et al.* 1987; Hillman 1988; Hillman *et al.* 1988; Jung and Carr 1977; Köhler *et al.* 1984; Kulkarni *et al.* 1984; Lasekan *et al.* 1999; Mimouni *et al.* 1993; Sellars *et al.* 1971; Steichen and Tsang 1987; Venkataraman *et al.* 1992). Two of the 13 studies reported significant decreases in growth measurements in infants fed soy formula when compared to infants fed casein- and rice-based hydrolyzed formulas (Agostoni *et al.* 2007) or compared to infants fed a milk-based formula (Cherry *et al.* 1968). In addition to these "limited" utility studies, there were a large number of "no utility" studies of small sample size included in the expert panel report that consistently reported similar growth trajectories of anthropometric measurements among the different infant feeding groups. Based on this overall pattern of response, the NTP concludes there is "some evidence of no adverse effects" on growth in healthy full-term infants (**Figure 2**).

It is worth noting that although all of the studies of gastrointestinal effects reviewed by the expert panel were classified as having "no utility," extensive reviews by the AAP and ESPGHAN have noted the possibility of adverse effects in a subset of infants with documented cow milk protein allergy (Agostoni *et al.* 2006; Bhatia and Greer 2008). Infants with documented cow milk protein-induced enteropathy or enterocolitis frequently are sensitive to soy protein and should not be given soy protein formulas. Instead, the recommendation is to provide formula derived from hydrolyzed protein or synthetic amino acids (Agostoni *et al.* 2007).

Reproductive System

The NTP ;considered the existing literature in humans "insufficient" for assessing impacts on the reproductive system from the use of soy infant formula (**Figure 2**); only three studies were considered by the expert panel to be of sufficient utility for assessing these types of effects (Boucher *et al.* 2008; Freni-Titulaer *et al.* 1986; Strom *et al.* 2001). The most comprehensive assessment of reproductive function of men and women following the consumption of soy formula as infants did not report significant impacts, but it also lacked sufficient power for several endpoints (i.e., cancer, reproductive organ disorders, hormonal disorders, libido dysfunction, sexual orientation, and birth defects in the offspring) to rule out increased risks (Strom *et al.* 2001). Two significant findings were reported in this study related to menstrual cycling in adult women who were fed soy formula during infancy. One was that women who had been given soy infant formula reported having longer menstrual periods (adjusted mean difference of 0.37 days; 95% CI, 0.06–0.68, P=0.02) and a soy infant formula-associated increase in the risk of experiencing extreme menstrual discomfort (unadjusted RR, 1.77; 95% CI, 1.04–3.00, P=0.04). However, these findings would not be considered statistically significant if a multiple comparison adjustment were applied to account for the number of hypothesis. The remaining two studies of "limited" utility dealt exclusively with an association of soy infant formula consumption and effects on the breast, i.e., premature thelarche (Freni-Titulaer *et al.* 1986) or risk of breast cancer in adulthood (Boucher *et al.* 2008). These two studies are discussed below in the context of other findings on the breast following consumption of soy formula during infancy.

Subsequent to the expert panel evaluation, a study was published that reported a 25% higher early uterine fibroid diagnosis (diagnosis by the age of 35) for women who reported being fed soy formula during infancy (relative risk=1.25, 95% confidence interval of 0.97 – 1.61) (D'Aloisio *et al.* 2010). There was also a higher risk of a similar magnitude in association with being fed soy formula within the first two months of life (adjusted RR=1.25; 95% CI: 0.90, 1.73). These findings were based on assessment of 19,972 non-Hispanic white women of 35 to 59 years of age at enrollment in the NIEHS Sister Study. The most common signs of fibroids are longer menstrual periods, heavy bleeding, and pelvic pain (Mayo Clinic), all of which were evaluated to some degree in the Strom *et al.* (2001) study. Indications of heavy bleeding were not observed in that study based on self-reported assessment of menstrual flow, but a significant association was reported between use of soy infant formula and longer menstrual periods (discussed above) based on assessment of the number of days requiring pads or tampons. With respect to pelvic pain, the other significant finding from Strom *et al.* (2001) was a higher reporting of extreme menstrual discomfort in women who consumed soy infant formula in infancy. The finding of higher risk of early uterine fibroid diagnosis associated with use of soy infant formula is also broadly consistent with reports that *in utero* exposure to the synthetic estrogen diethylstilbestrol is also associated with fibroid diagnosis (Baird and Newbold 2005; D'Aloisio *et al.* 2010) as well as histopathological findings reported in the uterus of adult mice treated with genistein

as neonates (Newbold *et al.* 2001). One limitation to the D'Alosio *et al.* (2010) study is the use of a self-administered family history questionnaire and dichotomous response ("ever" or "none" on soy infant formula feeding; "yes" or "no" on soy infant formula feeding ≤ 2 months of age) for assessing exposure to soy infant formula. The NTP agrees with the author's interpretation that the association with early diagnosis of uterine fibroids is interesting and needs to be replicated. Another observation from the NIEHS Sister Study, currently available only in abstract form, are findings that use of soy infant formula was associated with both higher odds of very early menarche (<11 yrs) and late menarche. (D'Aloisio *et al.* 2009).

In addition to the three studies considered of "limited" utility described above (Boucher *et al.* 2008; Freni-Titulaer *et al.* 1986; Strom *et al.* 2001), the expert panel evaluated four other studies of infants fed soy infant formula that included assessment of reproductive system development; however, these studies were considered to have "no utility" for the evaluation (Bernbaum *et al.* 2008; Giampietro *et al.* 2004 Zung, 2008 #2434; Gilchrist *et al.* 2009). The expert panel spent a considerable amount of time discussing the outcomes from two of these studies. One was a pilot study to identify estrogen responsive endpoints in infants (Bernbaum *et al.* 2008), and the other was an interim analysis from an ongoing prospective cohort design study (Gilchrist *et al.* 2009).

The pilot study by Berbaum *et al.* (2008) was conducted as part of the Study of Estrogen Activity and Development (SEAD), a series of mostly cross-sectional pilot studies designed to establish methods for future larger studies evaluating the estrogenic effects of soy infant formulas (or any putative estrogenic exposure) on the developing infant (*http://www.niehs.nih.gov/research/atniehs/labs/epi/studies/sead/index.cfm*)[7]. SEAD had a mixed, cross-sectional study design that included equal numbers of infants fed soy infant formula, cow milk formula, or breast milk. The pilot study evaluated breast and genital development in infants during the first 6 months of life, i.e., breast buds, breast adipose tissue, testicular volume and position, vaginal discharge, and cell maturation. Of these measurements, the authors considered measurement of breast buds and cell maturation of the vaginal wall to be the most valuable for evaluating exposures to compounds with estrogenic-like activity in humans. Breast bud diameter was maximal in the week after birth and smaller in older infants, both male and female, at 2 weeks to 6 months. The maturation index of cells of the vaginal wall was maximal in 1 week old infants and lowest at 1 month. Breast bud diameter and vaginal wall cell maturation index were considered the most estrogen-sensitive endpoints because they displayed a pattern of reversion during the period when infants would be withdrawing from the high maternal estrogen exposures that occur during pregnancy. While the authors very clearly described this study as a pilot and of too small a size to make

[7] Analysis of isoflavones in the blood, urine, and saliva from these children based on feeding regimen are presented in Cao *et al.* (2009). Other data from this pilot study have only appeared in abstract form and include characterization of sex hormones (Pediatric Academic Societies, 2007 meeting), thyroid hormones (*International Society for Environmental Epidemiology,* 2009 meeting), and ultrasound evaluation of breast, testes, ovary, thyroid, and uterus (Pediatric Academic Societies, 2006 meeting). Abstracts from the Pediatric Academic Societies meetings that mention the SEAD study and have not yet been presented in peer-reviewed publications are available at *http://www.pas-meeting.org/2009Baltimore/abstract_archives.asp*.

[8406.2] Umbach, D., Phillips,T., Davis,H., Archer, J., Ragan, B., Bernbaum, J., Rogan, W. (2007) Relationship of Endogenous Sex Hormones and Gonadotropins to Soy infant formula Diet in Infants;

[2757.8] Estroff, J., Parad R., Stroehla, B., Umbach, D., Walter Rogan, W. (2006) Developing Methods for Studying Estrogen-like Effects of Soy Isoflavones in Infants 3: Ultrasound

reliable inferences about feeding regimens, the trajectory of maturation index appeared to differ in the infants fed soy infant formula (P=0.07), such that these infants tended to have a higher maturation index at 3 to 6 months compared to infants fed breastmilk or a cow milk-based formula. Vaginal cell maturation indices are used as a measure of estrogen effects in adult women and have also been used in the diagnosis and evaluation of treatment for precocious puberty in girls [reviewed in Berbaum *et al.* (2008)]. The expert panel considered this pilot study of "no utility" for the evaluation given the variability observed and because the sample size was very small (once gender and age were considered) and thus underpowered statistically to detect any relevant associations. Based on the results of the pilot studies, a prospective study (Infant Feeding and Early Development, or IFED) of infants fed soy infant formula, cow milk formula, or breast milk (n=300; 50 boys and 50 girls in each feeding group) has been planned and will include assessment of the endpoints evaluated in the pilot studies as well as others that allow testing of additional hypotheses, e.g., altered response to vaccination, changes in play behavior, or language acquisition in toddlers. Recruitment for this prospective study, which will be carried out at the Children's Hospital of Philadelphia, is expected to begin in spring 2010.

The study by Gilchrist *et al.* (2009) was an interim report from a prospective, longitudinal study in children aged 2–3 months through 6 years who were breast-fed, cow milk formula-fed, or soy infant formula-fed as infants being conducted by the Arkansas Children's Nutrition Center (ACNC). The completed study will include assessments of growth, development, body composition, endocrine status, metabolism, organ development, brain development, cognitive function, language acquisition, and psychological development at 3, 6, 9, 12, and 18 months and at 2, 3, 4, 5, and 6 years. The interim examination of the data published by Gilchrest *et al.* (2009) summarized differences in hormone-sensitive organ size at 4 months of age in infants fed soy infant formula (SF) (n=39, 19 males and 20 females), milk formula (n=41, 18 males and 23 females), or breast milk (n=40, 20 males and 20 females) (Gilchrist *et al.* 2009). A major limitation in the study is the amount of cross-feeding that occurred in the cohort.8 All breastfed infants were stated to be exclusively fed breast milk the entire study time. Only 23% of infants in the SF group were exclusively fed soy infant formula from birth, 45% were switched to exclusive soy infant formula feeding within 4 weeks, and 32% were switched to soy infant formula between 4 and 8 weeks. Thus, the length of soy infant formula exposure varied from 2 to 4 months. Fifty-four percent of the infants in the milk formula group were stated to be exclusively fed milk formula from birth, 41% switched from breast milk to cow's milk formula within 4 weeks, and 5% switched between 4 and 8 weeks. At age 4 months, anthropometric measures (weight, length, and head circumference) were assessed using standardized methods, and body composition was assessed by air displacement plethysmography. Breast buds, uterus, ovaries, prostate and testicular volumes were measured by ultrasonography.

Gilchrist *et al.* (2009) concluded that the results did not support major diet-related differences in reproductive organ size as measured by ultrasound in infants at age 4 months, although there was some evidence that ovarian development might be advanced in milk formula-fed infants and that

[8] The Berbaum *et al.* (2008) study appears to have required stricter criteria for feeding regimen eligibility compared to Gilchrist *et al*. (2009). In Berbaum *et al.* (2008), breast milk and cow's milk regimens prohibited use of soy foods in baby's lifetime; however, infants in the soy infant formula group were allowed breast milk or cow milk while the baby was in the hospital just after birth. In older infants, ≥ 3 months, soy infant formula regimen must have been fed exclusively and continuously for at least two-thirds of the child's lifetime, including 2 weeks before the study examination.

testicular development might be slower in both milk formula and soy infant formula infants as compared with infants fed breast milk. The direction of effect on testicular volume was opposite of that reported by Tan *et al.* (2006) in a study of marmoset monkeys with seven sets of co-twins where one twin from each set was fed a cow milk-based formula as the control and the other twin was fed soy infant formula milk for 5–6 weeks during infancy (infants also nursed during this period).

With respect to future consideration of the cohort described in Gilchrist *et al.* (2009), the expert panel noted the benefit of longitudinal data in characterizing differences in developmental endpoints across the exposure groups as a valuable study design feature. However, when exposure is mixed due to the cross-feeding across groups, the effects may be attenuated or exaggerated which makes the results thus far of no utility. Given that the report by Gilchrist *et al.* (2009) is an interim report from an ongoing prospective study, the expert panel noted that the completed study would have greater value if continued recruitment did not permit such extensive dietary transitions or data are collected prior to these transitions. The NTP recognizes the value of the prospective studies being conducted through the Arkansas Children's Nutrition Center study (directed by Dr. Thomas Badger) and the Infant Feeding and Early Development study directed by Dr. Walter Rogan at the Children's Hospital in Philadelphia. Both are important studies as each is designed to address different aspects of the issue regarding potential health effects of soy infant formula. The Infant Feeding and Early Development study has more stringent criteria for designating an infant as "soy formula fed" and may be better able to address potential health effects in infants exclusively fed soy infant formula, while the Arkansas Children's Nutrition Center study may be a better indicator for infants who are cross-fed.

Effects on the Breasts

Seven studies evaluated by the expert panel included some assessment of the breast, either breast bud size in infants, age at breast development in girls, or risk of breast cancer in adulthood. Some of these studies were small in sample size or had other experimental features that resulted in their classification as "no utility" by the expert panel. However, the NTP considered findings from all of the studies for any overall pattern of response on breast development (**Table 3**) given that understanding possible effects on breast tissue, especially breast cancer risk, is of particular interest in the context of soy use, such as based on geographical differences in dietary ingestion of soy, e.g., Western versus Asian diets, or use of soy supplements.

One study assessed the association between use of soy infant formula in infancy and breast cancer in adulthood. Boucher *et al.* (2008) compared women with and without breast cancer and reported reduced, but non-significant, associations between soy infant formula intake and breast cancer: soy infant formula only during first 4 months of life: OR=0.42, 95% CI=0.13–1.40; soy infant formula only during 5–12 months of age: OR=0.59, 95% CI=0.18 – 1.90). Although non-significant, this pattern is consistent with conclusions from meta-analyses of limited human data and the animal model data (discussed below) that provide some support for a potential modestly protective effect for some soy or soy isoflavone exposures, e.g., childhood/adolescent exposure might have a small reduction in risk.

Other studies assessed breast bud development in infants or indication of premature thelarche, defined as breast development before the age of 8 without evidence of sexual hair development, estrogenization of vaginal mucosa, acceleration of linear growth, rapid bone maturation, adult body odor, or behavioral changes typical of puberty.

Table 3. Summary of Epidemiological Findings of Breast-Related Measures in Association with Use of Soy Infant Formula

Breast-Related Endpoint and Reference	*Study Design*	*Sample Size*	*Major Findings*	*Expert Panel's Utility Category*
Breast cancer: In adulthood Boucher *et al.* 2008	Population-based case-control design Association of breast cancer with type of milk consumed during infancy	• Adults with breast cancer (n=372) • Controls without breast cancer matched within 5-year age groups (n=356)	Non-significant suggestions of reduced risk: • soy infant formula only during first 4 months of life: *OR = 0.42, 95% CI = 0.13–1.40* • soy infant formula only during 5–12 months of age: *OR = 0.59, 95% CI = 0.18–1.90* (Multivariate analysis to control for possible confounding factors)	Limited utility
Breast development: Age when started to wear a bra Strom *et al.* 2001	Retrospective cohort study of adults who participated as infants in a non-randomized controlled feeding study	Adults fed during infancy: • SF (n=127) • MF (n=268)	No difference in unadjusted or adjusted means (SF=12.3 years versus MF=12.3 years) Multivariate analysis to control for possible confounding factors	Limited utility
Breast development: Premature thelarche Freni-Titulaer *et al.* 1986	Age-matched pair case-control study	• Girls with premature thelarche • Age-matched controls • (n=120 for each group in final analysis)	Premature thelarche before 2 years of age and consumption of SF: *OR = 2.7, 95% CI = 1.1–6.8* (Multivariate analysis to control for possible confounding factors)	Limited utility
Breast development: Breast bud diameter and palpable buds in infants from birth to 6 months Bernbaum *et al.* 2008	Mixed cross-sectional (Pilot study to identify techniques for assessing infants' responses to withdrawal from maternal estrogen)	• 37 male and 35 female infants <48 hr to 6 months • One-third of the children of each sex and age interval were fed BM, MF, or SF	↓ breast bud size and ↓ proportion of children with palpable buds during the 6-month period of assessment in both boys and girls; no obvious difference in pattern for infants in the SF group (statistical analyses not conducted to determine effects of feeding regimen)	No utility
Breast development: Presence or absence of breast buds in children ages 7–96 months Giampietro *et al.* 2004	Retrospective study	• 48 children (27 boys and 21 girls) exclusively fed SF for at least 6 months • Range of 6–82 months Median 12 months	None of the girls demonstrated clinical signs of precocious puberty None of the males showed gynecomastia (univariate analysis)	No utility

Table 3 (continued)

Breast-Related Endpoint and Reference	*Study Design*	*Sample Size*	*Major Findings*	*Expert Panel's Utility Category*
Breast development: Breast bud volume in 4-month old infants Gilchrist *et al.* 2009	Prospective longitudinal cohort study (Interim analysis)	• 20 boys and 20 girls in BM group • 18 boys and 23 girls in MF group • 19 boys and 20 girls in SF group	No effect on breast bud volume in boys or girls (univariate analysis)	No utility
Breast development: Prevalence of breast buds in female infants ages 3 – 24 months Zung *et al.* 2008	Cross-sectional	*Both years:* • 92 in SF group • 602 in a combined "milk" group of infants fed MF or BM *First year:* • 42 in SF, • 370 in "milk"' *Second year:* • 50 in SF • 232 in "milk"	• Breast buds more prevalent in 2nd year of life in infants fed SF vs. "milk" *(OR = 2.45 05% CI = 1.11 – 5.39)* • No differences in 1st year of life. • No differences in infants exclusively fed soy infant formula compared to those that had mixed feeding (univariate analysis)	No utility

Soy infant formula (SF); milk formula (MF); breast milk (BM).
Shaded cells are studies that have some limited utility.

One study of "limited utility" based on retrospective patient recall reported that use of soy infant formula may be associated with premature thelarche, or the start of breast development, before age 8 in girls, without other indications of sexual maturation (130 subjects from 552 potentially eligible girls) (Freni-Titulaer *et al.* 1986). Age-matched controls were recruited and parents were interviewed with regard to family history and possible exposures including the use of soy infant formula. Multivariate analysis did not show a significant relationship between premature thelarche and soy infant formula feeding except when the analysis was restricted to girls with onset of premature thelarche before 2 years of age (OR 2.7, 95% CI 1.1–6.8). Other significant factors included maternal ovarian cysts (OR 6.8, 95% CI 1.4–33.0) and consumption of chicken (OR 4.9, 95% CI 1.1–21.9). Consumption of corn was protective (OR 0.2, 95% CI 0.0–0.9). All other studies reporting on breast development in infants or young children were considered of "no utility" by the expert panel. The clinical or pathophysiological outcomes of premature thelarche are not clear. For example, a study by de Vries *et al.* (2009) suggests that premature thelarche does not predict precocious puberty. In this study, breast development and puberty were followed in 139 girls diagnosed with premature thelarche; it regressed in 50.8%, persisted in 36.3%, progressed in 3.2%, and had a cyclic course in 9.7%. With respect to age at diagnosis, progressive or cyclic course was more commonly found among girls presenting after 2 years (52.6%) compared with girls presenting at birth (13.0%) or at 1 to 24 months (3.8%). Precocious puberty occurred in 13% of girls and was not related to age at premature thelarche or clinical course.

The only other study reporting an association between soy infant formula and breast development reported an increased prevalence of breast buds in females during the second year of life (but not during the first year) (Zung *et al.* 2008), a finding that was interepreted by the authors as suggesting that soy phytoestrogens may have a "preserving" effect on breast tissue in infants. The authors also suggested that the lack of association during the first year could be a function of the high plasma levels of endogenous estrogens that infants have at that time, potentially masking any estrogenic effects of soy phytoestrogens. Giampietro *et al.* (2004) also looked at female infants during this age range, but reported no difference in breast bud prevalence in children ages 7–96 months. Gilchrist *et al.* (2009) also reported no differences in breast bud volume at 4 months of age in girls or boys in relation to feeding regimen and there were no apparent differences in pattern of breast bud development in girls or boys based on feeding regimen in the pilot data presented in Bernbaum *et al.* (2008). However, infants in both of these studies were assessed at ≤ 6 months which would limit the ability to identify any effect consistent with the "preserving" effect reported in Zung *et al.* (2008).

Thyroid

Although the expert panel considered the evidence insufficient to reach a conclusion on whether use of soy infant formula produces or does not produce adverse effects on thyroid function, they identified continued observational studies of thyroid function in infants fed soy infant formulas as a research need. This recommendation was based on case-studies for a special cohort of infants and children with congenital hypothyroidism (CH) fed soy infant formula who demonstrated a delay of thyroid stimulating hormone (TSH) levels returning to normal after adequate treatment; these children may need increased doses of levothyroxine (also called L-thyroxin) and closer follow-up. This conclusion is consistent with the recommendation of the New Zealand Ministry of Health that clinicians treating infants with hypothyroidism who consume soy-based infant formula closely monitor the doses of thyroxin required to maintain a euthyroid state (New Zealand Ministry of Health 1998). In addition, the New Zealand Ministry of Health recommends that clinicians treating children for medical conditions

who consume a soy-based infant formula be assessed for thyroid function if there are concerns for unsatisfactory growth and development.

In the 1950s and 1960s, cases of altered thyroid function, mostly goiter, were reported in infants fed soy infant formula at a time when the formula contained soy flour. The cases of goiter in infants were consistent with reports from the 1930s that rats fed soybeans developed goiters (reviewed in the UK Committee on Toxicity Report on Phytoestrogens and Health (2003), Fitzpatrick (2000), McCarrison (1933), Sharpless (1938), and Wilgus *et al.* (1941). The problem of goiter in infants fed soy infant formula was eliminated in 1959 by adding more iodine to the formulas and in the mid-1960s by replacing the high-fiber soy flour with soy protein isolate. Although the early reports of goiter in infants fed soy infant formula have mostly ceased since manufacturers began supplementing soy infant formula with iodine,[9] there have been reports that use of soy infant formula in infants with congenital hypothyroidism may decrease the effectiveness of thyroid hormone replacement therapy, i.e., L-thyroxin (Chorazy *et al.* 1995; Conrad *et al.* 2004; Jabbar *et al.* 1997). This effect has been attributed to fecal wastage with decreased enterohepatic circulation (Chorazy *et al.* 1995; Jabbar *et al.* 1997; Shepard 1960).

Laboratory Animal Studies

Only two studies have assessed the effects of direct ingestion of soy infant formula in laboratory animals during infancy. Thus, there is insufficient evidence to reach a conclusion on whether use of soy infant formula causes, or does not cause, developmental toxicity in animal models. The weight-of-evidence determinations presented in Figure 3 also include conclusions based on animal studies administering (1) the individual isoflavones found in soy infant formula, namely genistein; (2) diets with high isoflavone content compared to soy-free or low soy diets; and (3) soy protein isolate or mixtures of isoflavones (i.e., genistein and daidzein).

Weight of Evidence Conclusions Based on Animal Studies of Genistein, Daidzein, Equol, and Glycitein

The expert panel reviewed more than 120 laboratory animal studies involving treatment with genistein or other individual isoflavones in its evaluation of soy infant formula. Of these, 74 were considered to be of "limited" or "high" utility (see Expert Panel Report, Tables 154–156). Seventy of these studies involved treatment with genistein. Based on these studies, exposure to genistein produced *clear evidence* of adverse effects on the female reproductive system following treatment during development (**Figure 3**). Studies that demonstrated clear evidence of developmental toxicity for genistein involved treatment only during the period of lactation in rodents (PND 1–21) as well as multigenerational studies that included exposure during gestation, lactation, and post-weaning. A study of neonatal mice treated orally with genistin, the glucoside form of genistein that predominates in soy infant formula, also supports *clear evidence* of adverse effects on development of the female reproductive tract.

In contrast, only a very small number of studies have been published on the other isoflavones associated with soy infant formula, daidzein and its estrogenic metabolite equol, and no studies have evaluated the effects of developmental exposure to glycitein. Detection of typical estrogenic effects in these studies was mixed. For example, two of the four studies considered of "limited" utility by the expert panel

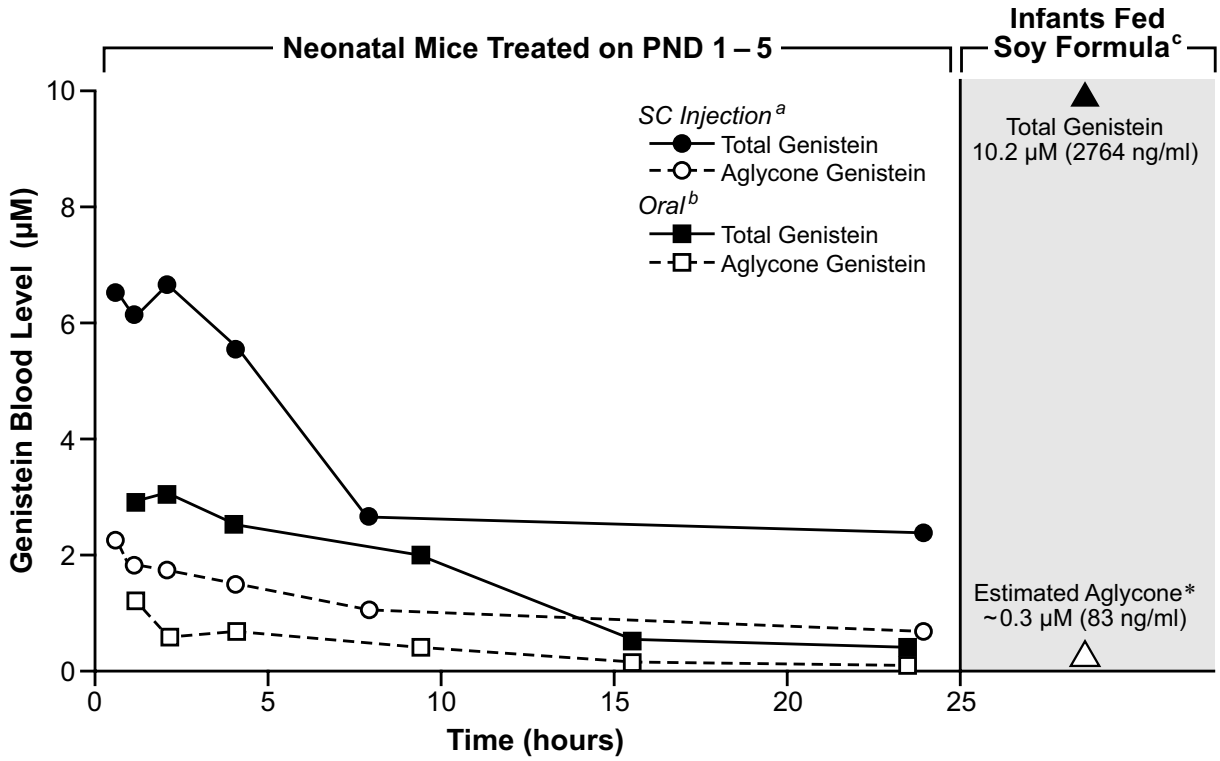
[9] In 1998, the New Zealand Ministry of Health noted one case report (Labib *et al.* 1989) on thyroid abnormalities associated with soy-based infant formula since iodine supplementation.

evaluated age at vaginal opening in rats treated with equol (Bateman and Patisaul 2009) or daidzein (Kouki *et al.* 2003) and neither reported the classic estrogenic effect of earlier age at opening. As part of a study that was primarily designed to assess the impact of *in utero* treatment of genistein and daidzein on uterine HOX10 gene expression, Akbas *et al*. (2007) evaluated uterotrophic response to these isoflavones in adult mice and did not detect an increase in uterine weight in mice treated with a single dose of 2 mg/kg of daidzein. Kouki *et al*. (2003) reported no effect on estrous cyclicity in rats treated by sc injection with ~19 mg daidzein/kg bw/day on PND 1–5. In contrast, treatment with the same dose levels of genistein caused the predicted estrogenic effect in all of these studies. However, two of the four studies did report effects that were consistent with an estrogenic effect. Bateman and Patisaul (2009) reported that sc injection of 10 mg equol/kg bw/day on PND 0–3 (day of birth, PND=0) in rats induced abnormal estrus cycles beginning at week 5 following vaginal opening. Genistein and estradiol benzoate also induced abnormal estrous cycles in this study. Kouki *et al*. (2003) reported a significant decrease in ovarian weight on PND 60 in rats treated by sc injection with ~19 mg daidzein/kg bw/day on PND 1–5; this same effect was observed in animals treated with estradiol or genistein. Based on the small number of studies and the inconsistent findings, the evidence is *insufficient* to determine whether daidzein or equol produces or does not produce developmental toxicity in laboratory animals.

"Clear Evidence" of Adverse Effects of Genistein/ Genistin in Studies Where Treatment Occurred During Lactation

Genistein induced adverse effects on the female reproductive tract when administered via sc injection during the period of lactation. Many of these studies were conducted by the same research group and used an experimental design where CD-1 mice were treated on PND 1–5 with genistein, typically by sc injection, and the reproductive system was assessed during late postnatal life or adulthood (Jefferson *et al.* 2009b; Jefferson *et al.* 2005; Newbold *et al.* 2001; Padilla-Banks *et al.* 2006). In young animals, neonatal treatment with 50 mg genistein/kg bw/day on PND 1–5 led to a higher incidence of multi-oocyte follicles on PND 4–6 (Jefferson *et al.* 2006) and PND 19 (Jefferson *et al.* 2002) compared to age-matched controls. In adulthood, the effects of neonatal exposure to 50 mg genistein/kg bw/day were manifest as a lower number of live pups per litter (Padilla-Banks *et al.* 2006), a lower number of implantation sites and corpora lutea (Jefferson *et al.* 2005), and a higher incidence of histomorphological changes of the reproductive tract (i.e., cystic ovaries, progressive proliferative lesions of the oviduct, cystic endometrial hyperplasia, and uterine carcinoma) (Newbold *et al.* 2001) relative to control females. In addition, the reproductive performance of the neonatally-treated mice was tested during adulthood and there was a significant negative trend for the number of dams with litters at PND 1–5 dose levels of 0, 0.5, 5, or 50 mg genistein/kg bw/day (Jefferson *et al.* 2005). In this study, there were no live litters produced by female mice treated with 50 mg genistein/kg bw/day as neonates and a reduction in the litter size in the females exposed to 0.5 and 5 mg genistein/kg bw/day on PND 1–5. Because the effects were more pronounced in animals at 6 months of age than at 2 or 4 months of age, the authors suggested that reproductive senescence may occur earlier in these animals as a result of the neonatal treatment (Jefferson *et al.* 2005). Finally, an alteration in the distribution of females in various stages of the estrous cycle was observed in animals exposed to ≥0.5 mg genistein/kg bw/day on PND 1–5 (Jefferson *et al.* 2005). Recently, oral treatment with 50 mg/kg bw/day of genistein on PND 1–5 in C57BL/6 mice was shown to cause effects that are consistent with the findings described above, including: an increased number of multi-oocyte follicle nests at PND 5 and 6 months of age, and a decrease in the number of estrous cycles during a 25-day period at 6 months of age (Cimafranca *et al.* 2010). However, there was no effect on fertility or age at vaginal opening in these animals.

Figure 4. Genistein Blood Levels in Infants Fed Soy Formula and Neonatal Mice Treated on PND 1 – 5 with 50 mg/kg/d Genistein by SC Injection[a] or Orally[b]

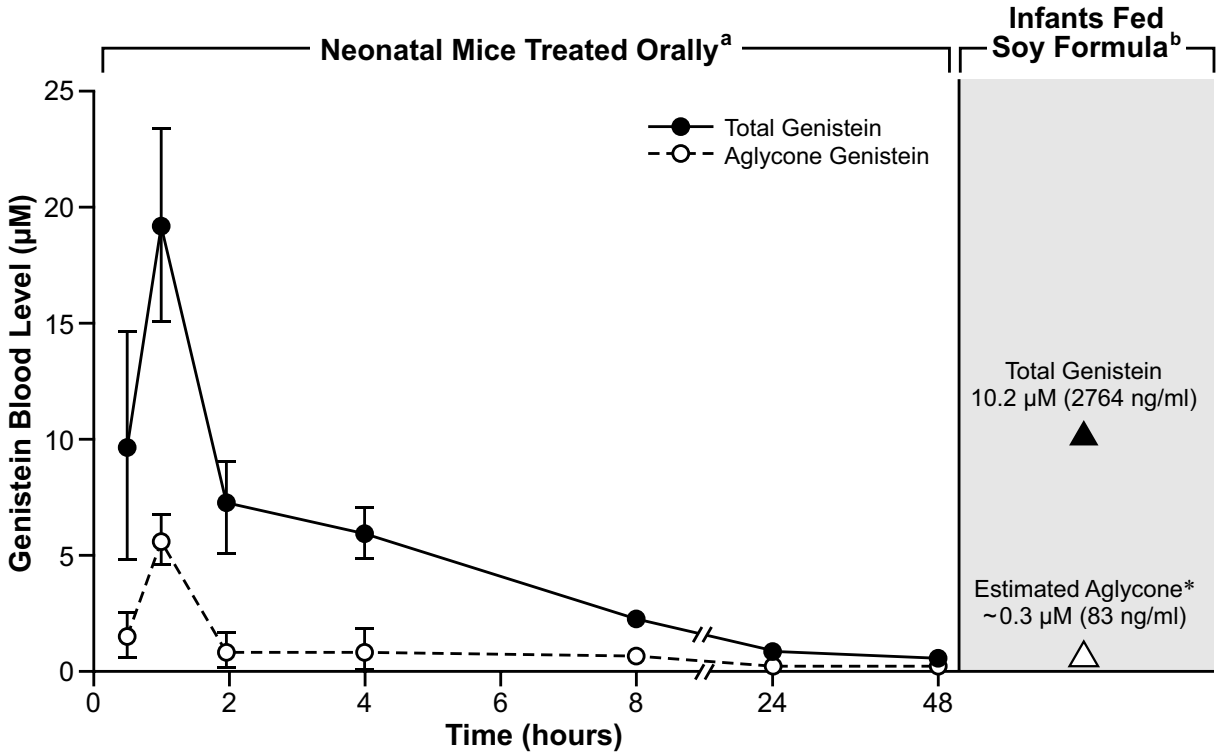


[a] CD-1 mice (Doerge *et al*., 2002).
[b] C47BL/6 mice (Cimafranca *et al.*, 2010).
[c] 95th percentile (Cao 2009, personal communication).
*The estimated aglycone is based on findings from adults that approximately 1 – 3% of total genistein is present in the unconjugated form (Setchell *et al.* 2001).

The NTP considered the blood profiles of unconjugated genistein as comparable in infant mice following treatment by the oral route or subcutaneous injection. This conclusion is most directly supported by comparing the blood levels of unconjugated genistein measured in neonatal mice following treatment on PND 1 – 5 with 50 mg/kg/d genistein by subcutaneous injection (Doerge *et al.* 2002) or orally (Cimafranca *et al.* 2010) (**Figure 4**).

Similar effects on female reproductive tract development were observed with oral treatment with genistin, the glycosylated form of genistin, directly to mouse neonates on PND 1 – 5 (Jefferson *et al.* 2009a). The effects of neonatal genistin exposure (expressed as aglycone equivalents) were manifested as a reduction in the number of live pups per dam at 37.5 mg/kg bw/day, altered estrous cyclicity at ≥25 mg/kg bw/day, impaired fertility (based on a reduction in the number of plug positive dams delivering pups), and a higher incidence of multi-oocyte follicles at PND 19 at ≥ 12.5 mg/kg bw/day. Interestingly, neonatal treatment with genistin administered orally on PND 1 – 5 elicited a greater uterotrophic response on PND 5 compared to oral administration of the comparable dose level of genistein. Genistin, expressed in aglycone equivalents, significantly increased uterine wet weight on PND 5 following treatment on PND 1 – 5 with 25 and 37.5 mg/kg bw/day relative to controls, whereas genistein did not produce any uterotrophic response at 37.5 mg/kg bw/day. In addition, although genistein induced

Figure 5. Genistein Blood Levels in Infants Fed Soy Formula and Neonatal CD-1 Mice Treated Orally with 37.5 mg/kg/d Genistin[a]



[a] Serum levels in mice from Jefferson et al. (2009a), doi: 10.1289/ehp.0900923.
[b] 95th percentile (Cao 2009, personal communication).
*The estimated aglycone is based on findings from adults that approximately 1–3% of total genistein is present in the unconjugated form (Setchell *et al.* 2001).

a significant uterotrophic response at a higher dose level (75 mg/kg bw/day), the magnitude of the response was smaller than that produced by genistin at lower administered dose levels.

The reason for the greater potency of genistin in the neonatal uterotropic assay is not entirely clear, but this finding is consistent with the much higher maximum blood levels of total genistein detected in the mice after treatment with 60 mg genistin/kg bw/day (37.5 mg genistein/kg bw/day when expressed as aglycone equivalents) or 37.5 mg/kg bw/day genistein (5189 versus 270.2 ng/ml, respectively). The level of the biologically active unconjugated aglycone form of genistein was similarly elevated in the mice treated with genistin compared to genistein. Blood levels of total genistein following this oral treatment with genistin were also higher than those reported by this research group in mice that were treated with 50 mg/kg bw/day genistein by sc injection on PND 1–5 (Doerge *et al.* 2002), the dose level and route of administration that caused many of the effects described above. This treatment resulted in a maximum serum concentration[10] of total genistein of 1350 ng/ml (5 µM), of which ~46% (621 ng/ml or 2.3 µM), was present as unconjugated genistein By way of comparison, blood levels of total genistein in infants fed soy infant formula at higher percentiles fall within the range of values reported by Jefferson *et al*. (2009a) for genistin-treated mice (**Figure 5**). The findings of higher blood levels following

[10] Sample collected 30 minutes following dose administration

genistin treatment are supported by a rat study by Kwon *et al.* (2007), which reported that genistin is more bioavailable than genistein possibly because it can be absorbed after hydrolysis to genistein, as well as absorbed in its intact form by passive transport across the membrane of the small intestine and via a sodium-dependent glucose transporter (SGLT1) in the small intestine brush border membrane.

Adverse effects on female reproductive development were also observed in rats exposed to genistein via sc injection or orally as neonates. These effects included earlier onset of vaginal opening and altered estrous cycling in Long Evans rats treated with 10 mg/kg bw/day by sc injection on PND 0–3 (day of birth, PND 0) (Bateman and Patisaul 2009); earlier onset of vaginal opening, altered estrous cyclicity, and a decrease in the number of corpora lutea in Wistar rats treated with 19 mg/kg bw/day on PND 1–5 by sc injection (Kouki *et al.* 2003); and decreased fertility, poly-ovular follicles in weanling females, and decreased number of implants per litter in Sprague Dawley rats treated orally with genistein at dose levels of 12.5 to 100 mg/kg bw on PND 1–5 (Nagao *et al.* 2001).

With respect to sexual maturation, an earlier onset of vaginal opening was observed in rodents exposed directly to genistein during the period of lactation. This effect was seen in CD-1 mice treated by sc injection on PND 15–18 with 10 mg/kg bw/day (3.1 day advance) (Nikaido *et al.* 2005) and rats treated by sc injection as neonates with 10 mg/kg bw/day (~2-day advance) (Bateman and Patisaul 2009) or ~19 mg/kg bw/day (7 day advance) (Kouki *et al.* 2003). A 4-day earlier onset of vaginal opening was also reported in a study where rats were treated by sc injection with 2 mg genistein/kg bw/day on PND 1–6, followed by oral treatment with 40 mg/kg bw/day on PND 7–21 (Lewis *et al.* 2003). An exception to this pattern was a delay in vaginal opening reported by Jefferson *et al.* (2009a) in CD-1 mice treated orally with 37.5 mg/kg bw genistin on PND 1–5; 50% of these females exhibited a 2-day delay and some did not have complete vaginal opening even 5 days after the last of the control animals. Also, Cimafranca *et al.* (2010) did not see any alterations in timing of vaginal opening in C57BL/6 mice orally treated with 50 mg/kg/d genistein as neonates.

"Clear Evidence" of Adverse Effects of Genistein in Studies with Gestational, Lactational, and Post-Weaning Treatment

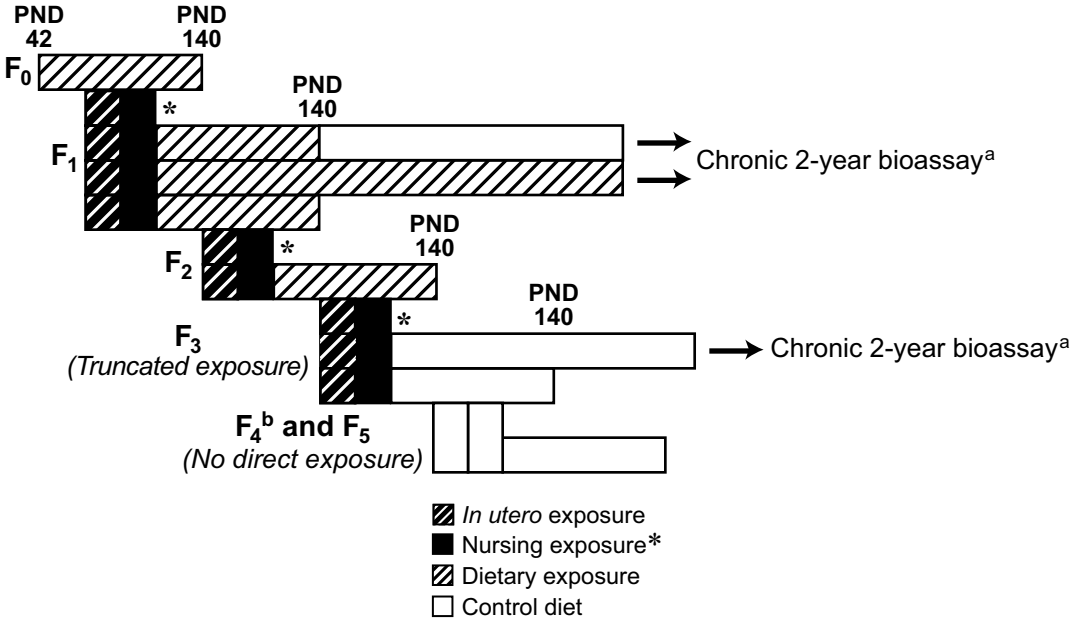
Clear evidence of adverse effects on the female reproductive tract was also observed in the NTP multigenerational reproductive toxicity study presented in NTP Technical Report 539 (NTP 2008a) where animals were fed dietary genistein at dose levels of 0, 5, 100, and 500 ppm. Additional data that assist in interpreting some of the effects observed in the multigenerational study are reported in NTP Technical Report 545, a chronic 2-year bioassay of genistein at these same dose levels where animals were treated from conception through weaning, 20 weeks of age, or until the end of the 2-year period (NTP 2008b). The study designs for these NTP Technical Reports are presented in **Figure 6**. A comparison of blood levels of genistein reported in human infants fed soy formula and in rats fed a diet containing 500 ppm geinistein (Chang 2000) is show in **Table 4**.

A number of effects related to growth and reproductive and developmental parameters were observed at 500 ppm (~35 mg/kg bw/day in males and ~51 mg/kg bw/day in females during the entire feeding period):

- **Reduced litter size:** Litter size of the 500 ppm group in the F_2 generation was significantly smaller compared to controls and the litter sizes in the F_1, F_2, and F_3 generations showed negative exposure concentration trends. These trends appeared to be largely determined by the 12% to 31% reduction

in litter size in the 500 ppm group of those generations. No other impacts on fertility and no histopathologic lesions were observed in females.

Figure 6. Study Designs of NTP Multigenerational Study (Technical Report 539) and Chronic Two-Year Bioassay (Technical Report 545)



* Negligible lactational transfer.

[a] Published in NTP Technical Report 545.

[b] F_4 generation was mated as F_0 to F_3 to produce F_5 litters.

Table 4. Genistein Blood Levels in Human Infants Fed Soy Formula and in Rats Fed a Diet of 500 ppm Genistein in a Multigenerational Study Design

Population		Blood Genistein		Reference
		Total Genistein, ng/ml	Aglycone, ng/ml (%)	
Infants fed soy infant formula	**95th percentile**	2764	27.6–82.9 (1–3)*	Personal communication; Cao 2009
Rats[a]	PND 140	**2145** (female) **1620** (male)	**21.5–107.3** (female) **16.2–81** (male) (1–5%)	Chang 2000
Infants fed soy infant formula	**75th percentile**	1455	14.6–43.7 (1–3)*	Cao 2009
	Median	891	8.9–26.7 (1–3)*	
Rats[a]	PND 21	**505** (female) **564** (male)	**5.1–25.3** (female) **5.6–28.2** (male) (1–5%)	Chang 2000

* The fraction of total genistein present as aglycone has not been established for human infants. The estimated range of 1–3% is based on data from adults (Setchell *et al.* 2001).

[a] Shaded cells represent studies of rats fed genistein.

- **Accelerated vaginal opening:** Females exposed to 500 ppm showed an accelerated time of vaginal opening (approximately 3 days) in the F_1 and F_2 generations, while the 5 ppm group showed an earlier time of vaginal opening (1.3 days) in the F_3 generation. Other studies administering genistein via the diet during gestation, lactation, or/and postnatal life also observed a younger age at vaginal opening (Casanova *et al.* 1999; Delclos *et al.* 2001; You *et al.* 2002a).

- **Altered estrous cyclicity:** When examined shortly after vaginal opening, estrous cycles of 500 ppm females in the F_1 and F_2 generations were significantly longer (approximately 3 days and 1 day, respectively) than those of their respective control groups. Other estrous cycle disturbances were confined to the 500 ppm group of the F_1 generation and included reduced time in proestrus and an increase in the number and percentage of aberrant cycles, with the exception of decreased time in diestrus for 100 ppm females in the F_4 generation. When the estrous cycles of animals were examined prior to termination from PND 130 – 140, the only significant effects were a decreased time in estrus and increased time in diestrus in 5 ppm females of the F_2 generation, and an increased number of abnormal cycles in 500 ppm females of the F_3 generation.

  Alterations in estrous cyclicity were also observed in the NTP 2-year chronic bioassay presented in NTP Technical Report 545 (NTP 2008b). In this study, animals were either (1) exposed from conception through 2 years, designated F_1 continuous, or F_1C; (2) exposed from conception through 20 weeks followed by control diet to 2 years, designated F_1 truncated at PND 140 or F_1T140; or (3) exposed from conception through weaning followed by control diet to 2 years, designated F_3 truncated at PND 21, or F_3T21. Estrous cycles were monitored starting at 5 months of age (~PND 150) to provide an estimate of when the animals began to show aberrant cycles, a condition known to precede reproductive senescence. An earlier onset of aberrant estrous cycles was observed at 500 ppm in the F_1C, F_1T140, and F_3T21 (with some evidence for effects at 5 or 100 ppm that were considered "marginal"). In all cases, the prevalent stage that caused the judgment of aberrant cycling was estrus, which appeared consistent with an acceleration of the senescence pattern typical of the Sprague-Dawley rat. While aberrant estrous cycles were not observed in PND 130 – 140 rats in the NTP multigenerational study, those females delivered and nursed litters shortly before evaluation, which may have had an impact on the observed cycle effects. The interpretation of earlier onset of reproductive senescence is consistent with the finding by Jefferson *et al.* (2005) related to the number of plug-positive mice that produced litters following treatment with genistein by sc injection on PND 1 – 5 (Jefferson *et al.* 2005). One hundred percent of plug-positive mice in the control group delivered litters when assessed at 2, 4, or 6 months of age, while the percentages decreased at these time points in animals treated with 0.5 mg/kg bw/day (100, 100, and 60%) or 5 mg/kg bw/day (75, 88, and 40%). Mouse dams exposed to the highest dose (50 mg/kg bw/day) on PND 1 – 5 did not produce litters even at 2 months of age.

- **Decreased body weight:** While pup birth weights were not significantly affected by genistein in the F_1 through F_4 generations (with the exception of 100 ppm males in the F_1 generation), both sexes in all generations showed depressed body weight gains during the pre-weaning period in the 500 ppm groups. Male pup pre-weaning body weight gains were also depressed in the 5 and 100 ppm groups in the F_1 generation. In the postweaning period, exposure to 500 ppm genistein reduced body weights predominantly in females of generations in which rats were ingesting the

compound throughout adulthood (F_0 through F_2). In the F_1 generation, postweaning body weights were reduced in all 100 and 500 ppm groups, with a more pronounced effect in the females. In the unexposed F_4 generation, female post-weaning body weight was also depressed, although to a lesser extent than in the earlier generations. Significant decreases in postweaning body weight in males were confined to the F_1 generation and were not seen in the similarly exposed F_2 generation. In the unexposed F_5 generation, pup birth weights in all exposed groups of both sexes were significantly lower than those in the controls, although this was interpreted as more likely a chance observation rather than a carryover effect from exposures in earlier generations. Other studies administering genistein via the diet during gestation, lactation or/and postnatal life also observed transient or permanent decreases in body weight (Awoniyi *et al.* 1998; Casanova *et al.* 1999; Delclos *et al.* 2001; Ferguson *et al.* 2009; Flynn *et al.* 2000; Masutomi *et al.* 2003; You *et al.* 2002a).

- **Decreased anogenital distance:** Male and female pups exposed to 500 ppm in the F_1 generation had slightly reduced anogenital distances relative to controls when analyzed with body weight as a covariate. Female pups also had reduced anogenital distances in the F_2 (500 ppm) and F_3 (100 ppm) generations, although the statistical significance was dependent on the analysis method applied.

- **Increased time to testicular descent:** Increased time to testicular descent was observed in 500 ppm males of the F_3 generation, although no other effects of genistein on male sexual development were reported.

Given the experimental design of multigenerational studies, it is impossible to determine whether the observed effects could be attributed to exposure during the period of lactation only. Exposures through placental transfer, lactational exposure, and feed ingestion could all have contributed to the reported findings. Studies conducted in conjunction with the NTP multigenerational study showed that genistein readily crosses the placenta; however, there was only limited lactational transfer via milk during nursing (Doerge *et al.* 2001). Specific findings were that fetal serum concentrations of total genistein were ~13- to 28-fold lower than maternal concentrations following treatment of Sprague-Dawley rat dams with a single gavage dose of 20, 34, or 75 mg/kg bw genistein on GD 20 or 21 (Doerge *et al.* 2001). However, the percent of genistein present as aglycone was greater in the fetuses at all dose levels (27 to 34%) compared to dams (8 to 18%), which resulted in blood levels of the biologically active genistein aglycone that were more similar between the fetus and dam as compared to the levels of total genistein. In contrast, there was limited transfer of genistein from dams to rat pups during lactation. Doerge *et al.* (2006) fed rat dams 500 ppm genistein (~51 mg/kg bw/day) in the diet starting immediately after parturition and assessed internal exposures to genistein in the pups during the early postnatal period when pups were exclusively nursing. The average serum levels of genistein measured on PND 10 from dams were ~2.6 times higher than milk levels of genistein collected on PND 7 (1.22 μM or 329.7 ng/ml compared to 0.47 μM or 127.0 ng/ml, respectively). On a daily intake basis, the estimated dose of genistein to dams from the feed was ~100 higher than to the neonates from milk (51 versus 0.51 mg/kg bw/day). Serum levels in the pups were ~ 30 times lower than in dams, 0.039 μM compared to 1.22 μM. The limited lactational transfer of genistein suggests that effects observed in the F_3 generation (treatment from conception to PND 21) were induced by *in utero* exposure or indicate a very sensitive response to neonatal exposure. With respect to support for sensitivity of response from lactational exposure, the body weight gain in pups from PND 7–10 was significantly lower for pups of genistein-fed dams (1.26 g) compared to pups from control dams (1.46 g) in the lactational transfer study (Doerge *et al.* 2006).

"Insufficient Evidence" for a Conclusion Based on Animal Studies of Soy Infant Formula

Only three publications report on the developmental effects of exposure to soy infant formula. One study in rats initiated treatment after the period of lactation and had several technical limitations that led the expert panel to consider it of "no utility" for the evaluation (Ashby *et al.* 2000). Two other publications reported data based on the same group of male marmosets treated during infancy and assessed either as juveniles (Sharpe *et al.* 2002) or adults (Tan *et al.* 2006), and both of these studies were considered of "limited" utility by the expert panel. While there were permanent effects on testicular cell populations (discussed further below), there were no obvious effects on reproductive function, i.e. fertility or permanent changes in testosterone levels. Overall, the evidence is insufficient to determine whether soy infant formula causes or does not cause developmental toxicity, due to the small number of studies, the limitations in their experimental designs, and failure to detect adverse functional effects.

Two studies reported the effects of feeding soy infant formula (versus standard cow milk formula) directly to infant marmosets (non-human primates) during the period of lactation (from PND 4 or PND 5 to PND 35 to PND 45; n=13 twin sets, plus four singletons) (Sharpe *et al.* 2002). Upon completion of treatment, the soy infant formula-fed males had significantly lower plasma testosterone levels than their cow milk formula-fed co-twins. Histopathological analysis on the testes of a subset of the co-twins on PND 35 to PND 45 revealed an increase in Leydig cell abundance per testes in the soy infant formula-fed marmosets compared to their cow milk formula–fed co-twin, in the absence of a significant change in testicular weight. A follow up study was conducted on the remaining animals when they were sexually mature (80 weeks of age or older; n=7 co-twin sets) (Tan *et al.* 2006). The males fed soy infant formula as infants had significantly heavier testes and an increase in the number of Leydig cells and Sertoli cells per testis as compared to cow milk formula-fed controls in the absence of a significant effect on timing of puberty, adult plasma testosterone levels, or fertility. The authors' suggest that the increase in testes weight was likely due to an increase in testicular cell populations. Tan *et al.* (2006) also state that the permanent change in Leydig and Sertoli cell populations may be due to compensation for Leydig cell failure following soy infant formula exposure during lactation. Since the animals were also allowed to nurse from their mothers, the authors suggest these studies may actually underestimate the effects of soy infant formula on human testicular development. In addition, the small number of animals studied and the lack of information on normal variability in the endpoints limit the utility of these studies.

"Insufficient Evidence" for a Conclusion Based on Animal Studies of Soy Protein Isolate, Soy-Based Diets, or Mixtures of Isoflavones[11]

Twenty-eight studies involving administration of soy protein isolate, soy-based diets, or mixtures of isoflavones to experimental animals were also judged by the expert panel to have utility in their evaluation. However, the heterogeneity of this literature in terms of administered form of soy, amount of

[11] The NTP considered whether information on domesticated animals, namely pigs, could be considered in reaching conclusions related to use of soy infant formula. However, the literature on livestock pigs was considered of very limited utility because (1) soy protein as an amino acid source is not typically introduced into the diet of pigs until after weaning, and (2) no specific safety assessments of soy isoflavones in diets fed to swine appear to have been conducted (December 3, 2009 public comment received from Dr. Hans H. Stein of the National Soybean Research Laboratory in Urbana, IL available at *http://cerhr.niehs.nih.gov/evals/genistein-soy/SoyFormulaUpdt/pubcom/HansStein12–02–2009.pdf*)

isoflavones, and differences in the experimental protocols hinders a clear interpretation of the toxicity literature. As a result, the NTP concurs with the expert panel that although some of the studies have identified potential developmental effects, these studies have yet to be replicated and overall provide insufficient evidence to conclude that soy isoflavone mixtures, including soy-based diets, produce or do not produce developmental toxicity in experimental animals.

Most of the developmental studies performed in rodents examined the effects of dietary soy products or soy-isoflavone preparations added to soy-free diets, and it is not clear to what extent these treatments are appropriate models for soy infant formula. In addition, the dietary interventions used in the experimental animal studies differ from one another, which can complicate interpretation of the literature. For example, one research group used a soy-based diet containing 102 mg genistein and 87 mg daidzin/kg diet (Masutomi *et al.* 2004) while another researcher used a phytoestrogen-free casein-based diet (AIN-93g) supplemented with soy protein isolate containing 286 mg genistein and 226 mg daidzein/kg diet (Ronis *et al.* 2009). There is also a paucity of dose-response studies of dietary soy product or soy-isoflavone preparations; for example, only one study evaluated by the expert panel utilized a soy-free diet supplemented with an isoflavone mixture giving rise to five different isoflavone intake levels (McVey *et al.* 2004a, b).

A generally consistent pattern of increased testicular weight was observed in rats and mice treated with soy diet or isoflavone supplements during gestation and lactation or continuous exposure, similar to the effect described above in marmosets treated with soy infant formula during infancy. Increased testicular weights were observed in 5/8 studies (Akingbemi *et al.* 2007; Mäkelä *et al.* 1995; McVey *et al.* 2004b; Odum *et al.* 2001; Ruhlen *et al.* 2008), while one study in rats reported a decrease (Atanassova *et al.* 2000) and two studies in rabbits observed no effect on testicular weight (Cardoso and Bao 2007, 2009). In particular, Akingbemi *et al.* (2007) reported an increase in testes weights (absolute and relative) on PND 28 rats with exposure to a soy-based diet supplemented with 5 – 1000 ppm and 50 – 1000 ppm isoflavones, respectively. At PND 90, absolute testes weights were decreased by the 50 – 1000 ppm isoflavone supplementation concurrent with an increase in serum testosterone levels at 1000 ppm isoflavone supplementation, relative to controls. McVey *et al.* (2004b) reported an increase in absolute testes weights at PND 28, but not at PND 120, in male rats continually exposure to soy-based diets containing from 36.1 to 1047 ppm isoflavones. Makela *et al.* (1995) observed increased testes weights in rats with continual exposure to a soybean diet at 12 months of age, but not at 2 months of age. Increased testes weights in rats were also observed by Ruhlen *et al.* (2008) at PND 90 and Odum *et al.* (2001) at PND 68 and PND 128 with continual exposure to soy-based diets relative to soy-free diets. In contrast, Atananssova *et al.* (2000) reported decreased testes weights in soy-diet control males relative to soy-free diet fed males. Interestingly, there was a decrease in spermatocyte nuclear volume per Sertoli Cell on PND 18 and PND 25 as well as a decrease in Sertoli Cell nuclear volume per testes at PND 18 in soy-diet control males relative to soy-free diet males (Atanassova *et al.* 2000).

There was less consistent data on timing of puberty and growth in rats and mice following exposure during gestation and lactation or continuous exposure to soy diet or supplements. Two of four studies reported a decrease in the age of vaginal opening of 5.9 days (Guerrero-Bosagna *et al.* 2008) or 1 day (Hakkak *et al.* 2000), and the remaining two studies reported an increase in age at vaginal opening (Odum *et al.* 2001; Ruhlen *et al.* 2008). Inconsistent effects were also reported for growth in rodents treated during development. Studies reported increases in body weight (Masutomi *et al.* 2004); both increases and decreases in body weight, depending on time at assessment (Akingbemi *et al.* 2007;

Mardon *et al.* 2008; Odum *et al.* 2001; Ruhlen *et al.* 2008); decreases in body weight (Atanassova *et al.* 2000; Gorski *et al.* 2006; Lephart *et al.* 2001; Lund *et al.* 2001); or no effect on body weight (McVey *et al.* 2004b; Pastuszewska *et al.* 2008).

Timing of Exposure and Effects on the Mammary Gland

Female

Timing of exposure during development appears to be important in determining the impact of soy isoflavones on mammary gland developmental pace and susceptibility to cancer risk. In general, there appears to be a lack of consensus in whether or not there is a "protective" effect or increased risk for hyperplasia/tumors following genistein treatment during the period of lactation. In an evaluation of three studies in rodents, (Cabanes *et al.* 2004; Hilakivi-Clarke *et al.* 1999b; Padilla-Banks *et al.* 2006), the common theme observed in the treated animals was that terminal end buds (TEBs) were in greater in number earlier in development and lower in number later in development when compared to controls, suggesting precocious development of the mammary epithelium. All of these studies utilized a sc injection of genistein directly to the pups at dose levels ranging from 0.7 – 50 mg/kg bw/day, and varied slightly in the timing of exposure, but all studies included at least 5 days of the nursing period. TEBs are considered to be very susceptible to chemical carcinogens, thus a decrease in the abundance of TEBs is an indicator of decreased cancer susceptibility (Russo *et al.* 1990). One of the three studies (Hilakivi-Clarke *et al.* 1999b) reported decreased multiplicity of tumors, but not incidence, in genistein-dosed rat offspring exposed to a chemical carcinogen, when compared to controls. Another study in rats (Cabanes *et al.* 2004) reported development of lobulo-alveolar structures, often correlated with decreased sensitivity to a carcinogen. However, a study in mice (Padilla-Banks *et al.* 2006) and a fourth study in rats (Foster *et al.* 2004) each observed hyperplasia and preneoplastic lesions in female offspring allowed to age normally following genistein exposure via sc injection to the pups during lactation. Some of these changes were similar to the types of changes normally observed in lactating animals (e.g., secretory changes in epithelial cells and lobular expansion) in addition to findings of increased incidences of atypical epithelial hyperplasia, microcalcifications, and *in situ* carcinoma (rats only) as compared to controls.

In contrast, exposure to genistein only during the period of gestation has been associated with effects on the pup mammary gland that are consistent with an increased susceptibility to mammary gland carcinogenesis. An increase in TEBs in female mice was observed on PND 35 and 45 following administration of genistein (~0.7 to 0.8 mg/kg bw/day) to the dam via sc injection on GD 15–20 (Hilakivi-Clarke *et al.* 1998). In another publication, this research group reported an increased incidence of mammary gland tumors in rats following dimethylbenzanthracene (DMBA) treatment on PND 50, following gestational exposure on GD 15–20 (~0.1 or ~1.5 mg/kg bw/day via sc injection to dams, but not ~0.5 mg/kg bw/day)(Hilakivi-Clarke *et al.* 1999a).

The NTP conducted a 2-year cancer bioassay of genistein that included a group of rats exposed via diet beginning with conception throughout life (National Toxicology Program (NTP) 2008) (**Figure 6**). There was some evidence of carcinogenicity based on an increased incidence of mammary gland adenoma or adenocarcinoma (combined) and pituitary gland neoplasms in females. The effects of genistein on the mammary gland were less clear with shorter periods of exposure, and equivocal evidence of mammary gland adenomas or adenocarcinomas was reported for females exposed from conception to weaning or conception to PND 140. In addition, there were conflicting results from two

studies with dietary exposure to genistein: one study using only prenatal exposure reported an increase in the number of TEBs at 8 weeks of age and a higher incidence of chemically-induced mammary tumors, but no changes in latency to tumors or multiplicity (Hilakivi-Clarke *et al.* 2002), and another study (Fritz *et al.* 1998) reported a persistent decrease in TEBs leading to a reduced tumor multiplicity and no change in tumor latency following gestational and lactational genistein exposures (incidence was not reported). Two common threads were apparent: developmental timing of genistein exposure was related to TEB versus mature duct end numbers and the level of TEBs present at the time of carcinogen exposure was related to number of tumors.

Exposure to dietary soy protein isolate appears to have a protective effect on female mammary gland development based on three rodent studies evaluating the effects reported for the abundance of TEBs or response to a chemical carcinogen challenge. In two studies, soy protein isolate was administered in diet to rats from preconception (Hakkak *et al.* 2000) and/or during pregnancy, lactation, and throughout life of the F_1 female offspring (Simmen *et al.* 2005). In both of these experiments, F_1 rats exposed to soy protein isolate displayed a longer latency to develop mammary gland tumors and a lower incidence of females with at least one mammary gland tumor following exposure to a chemical carcinogen on PND 50. Thomsen *et al.* (2006) administered a soy protein isolate in diet to mice during lactation or during lactation and throughout adulthood. They reported exposure to soy protein isolate during lactation increased the number of TEBs immediately after weaning (PND 28) compared to controls. On PND 42–43, the female rats continually exposed to soy protein isolate had a lower number of TEBs and on PNDs 70–73, there was no treatment difference in the number of TEBs. The authors speculated that treatment enhanced normal development and that the effects of treatment on tumor susceptibility may depend on the timing of exposure, such that a protective effect may be expected if carcinogenic insult is initiated late in puberty, i.e., PND 42–43, versus at an earlier point in development.

Male

One of the most consistent findings of the NTP studies was morphological changes in the mammary gland of male rats (Latendresse *et al.* 2009; 2008a). In the NTP perinatal dose selection study for genistein that tested dose levels of 5, 25, 100, 250, 625, and 1,250 ppm, an increased incidence of mammary gland hypertrophy was observed in males at ≥25 ppm and hyperplasia at ≥250 ppm. In a multigenerational evaluation of 0, 5, 100, or 500 ppm genistein (Latendresse *et al.* 2009), the incidence of mammary gland alveolar/ductal hyperplasia was significantly higher in 500 ppm males in the F_0 through F_2 generations and in 100 ppm males in the F_1 and F_2 generations. In the F_3 generation, a significant, positive, linear, exposure- concentration trend in the incidences of mammary gland hyperplasia occurred, but no exposed group differed significantly from controls in pairwise comparisons. Both developmental and adult exposures contributed to the maintenance of these effects. More dramatic effects of genistein on the incidences of male mammary gland hyperplasia were observed in the continuously exposed F_1 and F_2 generations as compared to the late adolescent and adult exposures of the F_0 generation and the pre-weaning-only exposure of the F_3 generation. Mammary gland hyperplasia was absent in males not directly or indirectly exposed to genistein (F_4 generation)(Latendresse *et al.* 2009; 2008a).

Mammary gland hyperplasia was also observed in the NTP 2-year chronic study at a lower incidence compared to the multigenerational study. In the 500 ppm dose group of the chronic study, the proportion of male mammary glands having hyperplasia (ductal and alveolar combined) was 19% of the

F_1C (exposed conception to 2 yr) and 20% of the F_1T140 (exposed conception to 140d) (Latendresse *et al.* 2009). In the multigenerational study, the incidence of mammary gland hyperplasia at 500 ppm was 60% in the F_1 males and 72% in the F_2 males (Latendresse *et al.* 2009). There was no clear evidence of progression of male mammary gland hyperplasia to neoplasia in the chronic study; i.e., there was "no evidence" of carcinogenicity activity in males of any generation for mammary gland or other tissue. Based on these data, Latendresse *et al.* (2009) concluded that the decline in incidence of mammary hyperplasia observed in the NTP chronic study was most likely due to regression of hyperplasia and glandular involution. Three other studies of dietary exposure during gestation and lactation or continuous exposure in male rats have reported an increase in mammary gland branching and epithelial cell proliferation (You *et al.* 2002a); an increase in mammary gland branching, TEBs, and lateral buds in male rats (You *et al.* 2002b); and an increase in size and tissue density of the mammary glands (Wang *et al.* 2006).

Consideration of Equol Production

The metabolic profile of daidzein varies in humans with 30 to 50% of individuals being classified as equol producers, and some individuals producing little or no equol, presumably due to differences in microbial factors, dietary consumption, lifestyles, or genetic factors (Atkinson *et al.* 2008a) (see Section 2.1.1.2 of the final expert panel report for additional discussion). Human infants are generally considered less able to produce equol compared to adults due to immaturity in gut microflora and/or underdeveloped metabolic capacity (Setchell *et al.* 1997). The expert panel considered the issue of equol production and concluded that rodent and monkey models receiving soy infant formula or other isoflavone mixture that included daidzein were relevant for humans because: (1) daidzein has estrogenic activity of its own and (2) some portion of human infants produce equol. The NTP concurs with this conclusion but recognizes that additional *in vivo* studies specifically designed to address the interactions between various soy isoflavones would be useful.

Equol is an estrogenic metabolite of daidzein with *in vitro*-based estimates of estrogenic potency that are generally intermediate between daidzein and genistein, e.g., **Table 5**. Overall, equol elicits estrogenic responses based on *in vivo* studies using classic measures of estrogenicity, although some

Table 5. Comparison of In Vitro Measures of Isoflavone Estrogenicity

Compound	Relative Binding Affinity, % [a]			Relative Estrogenic Activities		
	ERα	ERβ	β/α	ER Binding [b]	Yeast Transactivation [b]	E-screen [b]
E_2	100	100	1	++++	++++	++++
Genistein	2.07	14.8	7.1	+++	++++	+++
Daidzein	0.55	0.46	0.8	++	++	++
Equol	1.70	4.45	2.6	++	+++	++++
Glycitein	0.32	0.44	1.4	++	N.D.	++

[a] Relative binding affinity = (IC_{50} of E_2) ÷ (IC_{50} of test compound) × 100.
[b] Based on comparisons to E_2 alone: ++++ (≥ 100%), +++ (66%–99%), ++ (33%–66%), + (1%–33%). Potency estimates for ER binding were based on binding data for at least one ER type.
N.D. = Not determined.
From Table 1 in Choi *et al.* (2008).

studies suggest that equol may not be exerting these effects with a potency predicted from the *in vitro* studies (Bateman and Patisaul 2009; Breinholt *et al.* 2000; Medlock *et al.* 1995; Nielsen *et al.* 2009; Rachon *et al.* 2007; Selvaraj *et al.* 2004); see also Expert Panel Report, Section 2.2.9.2. For example, neonatal treatment with 10 mg genistein/kg bw/day by sc injection caused a ~2-day advancement in the day of vaginal opening, while there was no effect in animals treated with the same dose level of equol (Bateman and Patisaul 2009). However, the estrous cycles of these animals were significantly altered and less than 30% of females in both groups displayed regular estrous cycles (most animals were in persistent estrus or diestrus) by 10 weeks of age. Kouki *et al.* (2003) found less indication for estrogenic activity of daidzein compared to genistein in a study that compared the effects of neonatal treatment with ~ 19 mg/kg bw/day of either isoflavone (by sc injection). Estrogenic responses reported for genistein, but not detected for daidzein, included earlier onset of vaginal opening, persistent or prolonged estrous, loss of corpora lutea, and reduced lordosis quotient in female rats. Allred *et al.* (2005) reported that a smaller percentage of equol is circulating in the unconjugated form compared to genistein following oral exposure and suggested this may contribute to a reduced *in vivo* potency relative to *in vitro* predictions. In this study, the percentage of genistein present as aglycone (9%) was higher than the percentage of equol present as aglycone (1%) following ingestion of a soy flour diet in female Balb/c mice.

Assessment of other non-estrogenic endpoints leads to similar conclusions. Studies, mostly *in vitro*, have also examined effects of soy isoflavones on endpoints such as: effects on bone, cardiovascular/lipid regulation, cell growth, inflammation, immunity, and neurology (Expert Panel Report, Table 78). Of the 77 studies that presented data on these endpoints, the majority reported a similar pattern of relative ranking of genistein ≥ equol > daidzein based on magnitude of effect or relative potency. Across these studies, genistein was more potent than equol or daidzein in 60 of approximately 117 endpoints examined. The relative effects for all three isoflavones were similar in another 52 of these endpoints. Daidzein or equol caused a greater effect as compared to genistein for only five endpoints. It is worth noting that 16 of these studies also reported that genistein inhibited tyrosine kinase activity, while inhibition of this enzyme by daidzein was not observed. The tyrosine kinase activity data suggest that the effects of genistein could be due in part to a non-estrogen receptor mode of action. In all cases where an effect was observed, the isoflavones acted in the same direction (e.g., genistein and daidzein both inhibited bone resorption (Blair *et al.* 1996)). Collectively, these data do not support the notion that daidzein or equol markedly "offset" genistein activity.

One factor in interpreting the isoflavone literature is consideration of species differences in the ability to produce equol, an estrogenic metabolite of daidzein. It is generally accepted that a greater proportion of rodents and monkeys metabolize daidzein to equol compared to humans, at least in adulthood. The metabolic profile of daidzein varies in humans with some individuals producing little or no equol, presumably due to differences in microbial factors, dietary consumption, lifestyles, or genetic factors (Atkinson *et al.* 2008a). Human infants are generally considered less able to produce equol compared to adults due to immaturity in gut microflora and/or underdeveloped metabolic capacity (Setchell *et al.* 1997). No information has been published on the equol production capacity for infant rodents or monkeys, but the same pattern observed in human infants also appears to hold true for pigs. Gu *et al*. (2006) did not detect equol in the sera of one month old piglets fed ~8.6 mg/kg bw daidzein since infancy. In contrast, other studies have reported equol in the serum of older pigs (Farmer *et al.* 2010; Kuhn *et al.* 2004; Walsh *et al.* 2009).

The species differences in daidzein metabolism are not considered a significant factor in rodent studies where only genistein was administered and animals were fed a soy-free- or low-phytoestrogen diet. However, it can complicate the interpretation of studies that include daidzein for reaching conclusions on potential effects in human infants fed soy infant formula. One concern is that use of rodents or monkeys as animal models may "overestimate" the potential health risk to human infants fed soy infant formula. A negating effect of daidzein and/or equol on estrogenic effects of genistein is not generally predicted unless perhaps the binding of less potent isoflavone, i.e., daidzein, to estrogen receptors limits the access of genistein to those receptors. However, this would only make sense conceptually if the relative concentrations of the weak binders were much higher than concentrations of genistein, and they are not.

Based on detection frequency, the percentage of infants with detectable levels of equol in urine or plasma is similar to the percentage of adults considered to be "equol producers." Equol was detected in the urine of 25% of 4–6 month old infants (Hoey *et al.* 2004) and in the plasma of 4 of 7 (57%) 4-month old infants fed soy infant formula (Setchell *et al.* 1997), values that are comparable to the frequently cited range of 30–50% of adults considered to be equol producers (Atkinson *et al.* 2008a; Atkinson *et al.* 2008b; Bolca *et al.* 2007; Hall *et al.* 2007; Setchell *et al.* 2003). In a larger sample, Cao *et al.* (2009) were not able to detect equol in the blood (n= 27) or saliva (n=120) of infants aged 0 to 12 months on a soy infant formula diet for at least two weeks, although it was detectable in the urine of a small proportion, 6 of 124 (5%), of infants. One reason why equol might not have been detected in the Cao *et al.* (2009) study is because of the relatively high limit of detection. The mean plasma concentration of equol measured in soy formula fed infants by Setchell *et al.* (1997) was ~ 2 ng/ml (range across infants in all feeding groups was <LOD to ~5.5 ng/ml) while the limit of detection in whole blood for equol in Cao *et al.* (2009) was 12 ng/ml.

Both Setchell *et al.* (1997) and Cao *et al.* (2009) reported detecting equol in a greater proportion of infants fed cow milk-based formula compared to other feeding methods. In Setchell *et al.* (1997), 100% of infants fed a cow milk-based formula had detectable plasma levels of equol with a peak level up to 2 orders of magnitude higher than in infants fed soy-based formula. In contrast, equol was only detected in 4 of 7 (57%) infants fed soy infant formula and 1 of 7 (14%) breastfed infants. In Cao *et al.* (2009) equol was also detected in a higher percentage, 22%, of infants fed a cow milk-based formula compared to those fed soy infant formula (5%) or breast milk (2%), although the geometric means of urinary equol in the infants were comparable between feeding regimens (soy infant formula, cow milk formula, and breast milk were 2.3 ng/ml, 2.4 ng/ml, and 1.7 ng/ml equol, respectively). The finding of equol being more readily detected in infants fed a cow milk-based formula is not unexpected given that cows can produce equol from either the formononetin found in red clover or daidzein found in soy (King *et al.* 1998). There are also data suggesting that equol concentrations may be higher in organic milk products presumably because organic dairy cows eat more forage legumes compared to conventionally raised cows (Hoikkala *et al.* 2007).

Of the infants who do produce equol, they do not seem to produce equol to the same extent as adults. This conclusion is based on the most recent CDC data from NHANES. The geometric mean (10th – 90th percentile) of equol detected in urine for people aged 6 years and older was 8.77 µg/L (<LOD – 38.5) (U.S. Centers for Disease Control and Prevention 2008). This value is approximately 3.7 to 5.2-fold higher than urinary concentrations of equol measured in infants by the CDC and reported in Cao *et al.* (2009), which included infants fed soy infant formula who were exposed to higher daidzein levels than older children and adults.

Limitations of Studies that Only Administer Genistein

A major limitation in extrapolating the results of the genistein-only studies in laboratory animals that presented evidence of development toxicity to humans fed soy infant formula is the uncertainty on whether another component of soy infant formula, either isoflavone or other, could act to dampen the effects of genistein. As discussed above, a "negating" effect of daidzein or equol on genistein would not be predicted given that they all exhibit estrogenic activity; the prediction would be an exacerbation of estrogenic response. However, to date, these predictions have not been tested for the endpoints described above that present "clear evidence" of adverse effect for genistein, i.e., decreased in litter size, altered estrous cyclicity, etc.

In addition, it is also theoretically possible that non-isoflavone components of soy infant formula may alter the biological activity of the soy isoflavones. However, assessing such an interaction is complicated from an experimental design perspective. Treatment of infant animals with soy infant formula in an "off the shelf" preparation administered in an amount relevant for humans is quite challenging from a logistical perspective. Oral treatment with soy infant formula at levels that are comparable to intakes for human infants on a body weight-corrected basis would require that neonatal rodents be treated more than 15 times a day. In addition, neonatal animals need to nurse and interact with their mothers along with ingesting soy infant formula; therefore it is unlikely that sufficient soy infant formula could be administered to a laboratory animal at the concentration and volume (corrected for body weight) that is administered to a human infant. For example, the marmoset monkeys discussed in Sharpe *et al.* (2002) and Tan *et al.* (2006) were only fed soy infant formula 3 or 4 times a day during an 8-hour period on the weekdays and 1 or 2 times a day during a 2-hour period on weekends. At other times, the infant marmosets were with their mothers and free to nurse. On a volume-ingested basis corrected for body weight, the marmosets consumed approximately half the volume of 1-month old human infants exclusively fed soy infant formula, ~0.1 L/kg bw versus ~0.2 L/kg bw. The estimated intake of total isoflavones in the marmosets, 1.6–3.5 mg/kg bw/day, was approximately 20 to 85% of the estimated intake in human infants at 1-month old.

Although it may not be possible to administer infant laboratory animals a soy formula preparation that directly models human infant exposure, the NTP believes that utilization of the genistein/genistin-only studies in laboratory animals would be enhanced if the adverse findings (e.g., decreased litter size, altered estrous cyclicity, early onset of vaginal opening) were also observed following co-treatment with other soy isoflavones such as daidzein.

SHOULD FEEDING INFANTS SOY INFANT FORMULA CAUSE CONCERN

Infants fed soy infant formula are reported to consume as much as 6.2 mg/kg bw/day of total genistein, thus a 5 kg infant would consume ~30 mg/day of total genistein. Blood levels of total genistein in infants fed a soy infant formula diet can exceed those reported in young rats or mice treated with genistein during development at dose levels that produced adverse effects, i.e., early onset of sexual maturation, altered estrous cyclicity and decreased litter size (**Table 6**). While these types of adverse effects have not been reported in humans during 60 years of soy infant formula usage, adequate studies of the reproductive system have also not been conducted on girls or women following use of soy infant formula during infancy. Thus, the data in humans are not sufficient to dismiss the possibility of subtle or long-term adverse health effects in these infants.

Table 6. Summary of Blood Levels of Genistein in Human Infants Fed Soy Infant Formula and Laboratory Animals Treated with Genistein/Genistin, and Associated Effects Observed in Laboratory Animals

Blood Genistein		Description of Exposure Studies	Associated Effects Observed in Laboratory Animals
Total Genistein, ng/ml	Aglycone, ng/ml (%)		
5189, C_{max}	**1513, C_{max}** (29%)	Female mice on PND5 following oral treatment with 37.5 mg/kg bw/day genistin expressed in aglycone equivalents on PND 1–5. **Jefferson *et al.* 2009a**	• Abnormal estrus cyclicity • Decrease in litter size • Altered ovarian differentiation • Delayed vaginal opening • Delayed parturition **Jefferson *et al.* 2009a**
3563	**35.6–106.9** (1–3%)*	Infants fed soy infant formula, 99th percentile **Personal communication, Dr. Yang Cao, NIEHS**	
2764	**27.6–82.9** (1–3%)*	Infants fed soy infant formula, 95th percentile **Personal communication, Dr. Yang Cao, NIEHS**	
2145 (Female, PND140) **1620** (Male, PND140)	**21.5–107.3** (Female) **16.2–81** (Male) (1–5%)	Rats treated with genistein via the dam during gestation and lactation and directly through the diet after weaning with 500 ppm genistein average dose of ~35 mg/kg bw/day in males to 51 mg/kg bw/day in females during the entire feeding period. **Chang *et al.* 2000**	• Reduced litter size • Decreased body weight • Accelerated vaginal opening • Altered estrous cyclicity • Delayed testicular descent • Mammary gland hyperplasia in males **NTP 2008a**
1837, C_{max}	**575, C_{max}** (31.3%)	Female mice on PND5 following SC injection of 50 mg/kg bw/day genistein on PND1–5. **Doerge *et al.* 2002**	Increased incidence of multi-oocyte follicles **Jefferson *et al.* 2006; Jefferson *et al.* 2002** Lower number of live pups per litter **Jefferson *et al.* 2005; Padilla-Banks *et al.* 2006** Lower number of implantation sites & corpora lutea **Jefferson *et al.* 2005** Higher incidence of histomorphological changes of the reproductive tract: • cystic ovaries, • progressive proliferative lesions of the oviduct • cystic endometrial hyperplasia • uterine carcinoma **Newbold *et al.* 2001**

Table 6 (continued)

Blood Genistein		*Description of Exposure Studies*	*Associated Effects Observed in Laboratory Animals*
Total Genistein, ng/ml	*Aglycone, ng/ml (%)*		
1455	**14.6–43.7** (1–3%)*	Infants fed soy infant formula, 75th percentile **Cao *et al.* 2009**	
891	**8.9–26.7** (1–3%)*	Infants fed soy infant formula, median **Cao *et al.* 2009**	
757	**7.6–22.7** (1–3%)*	Infants fed soy infant formula, geometric mean **Cao *et al.* 2009**	
505 (Female, PND21) **564** (Male, PND21)	**5.1–25.3** (female) **5.6–28.2** (male) (1–5%)	Rats treated with genistein via the dam during gestation and lactation and directly through the diet after weaning with 500 ppm genistein. **Chang *et al.* 2000**. Average dose of ~35 mg/kg bw/day in males to 51 mg/kg bw/day in females during the entire feeding period. **NTP 2008a**	• Reduced litter size • Decreased body weight • Accelerated vaginal opening • Altered estrous cyclicity • Delayed testicular descent • Mammary gland hyperplasia in males **NTP 2008a**

*The fraction of total genistein present as aglycone has not been established for human infants. The estimated range of 1–3% is based on data from adults (Setchell *et al.* 2001).

Shaded cells are studies of infants fed soy infant formula.

In a study of 27 infants fed soy infant formula, the median serum level of total genistein was 890 ng/ml, with serum levels of total genistein reaching 1455 ng/ml at the 75th percentile (Cao *et al.* 2009) and 2763.8 at the 95th percentile (personal communication with Dr. Yang Cao, NIEHS). These blood levels in infants can exceed maximum concentrations of total genistein associated with dose levels of genistein that caused adverse developmental effects in rodents. Specifically, the maximum blood level of total genistein measured in female mice following daily sc injection of 50 mg/kg bw/day genistein on PND 1–5 was 1837 ng/ml or 6.8 µM (Doerge *et al.* 2002). A number of adverse effects on the female reproductive tract were reported in other studies that used this treatment protocol, including increased incidence of multi-oocyte follicles (Jefferson *et al.* 2006; Jefferson *et al.* 2002), lower number of live pups per litter (Jefferson *et al.* 2005; Padilla-Banks *et al.* 2006), lower number of implantation sites and corpora lutea (Jefferson *et al.* 2005), and higher incidence of histomorphological changes of the reproductive tract (i.e., cystic ovaries, progressive proliferative lesions of the oviduct, cystic endometrial hyperplasia, and uterine carcinoma) (Newbold *et al.* 2001). Similarly, blood levels of total genistein measured in human infants fed soy infant formula can exceed levels of total genistein measured in the NTP multigenerational study in rats on PND 21 and PND 140 following dietary treatment with 500 ppm (~35–51 mg/kg bw/day) of genistein (Chang *et al.* 2000).[12] Effects observed at the 500 ppm dose level included reduced litter size, decreased body weight, accelerated vaginal opening, altered estrous cyclicity, delayed testicular descent, and mammary gland hyperplasia in males (NTP 2008a) (**Table 6**).

Comparisons based on blood levels of unconjugated genistein between humans and rodents are more difficult because only total genistein was measured in the infants fed soy formula (Cao *et al.* 2009). However, in adults approximately 1–3% of total genistein is present in the unconjugated form (Setchell *et al.* 2001). If this range is applied to the blood levels of total genistein measured in infants fed soy formula, then the estimated levels of unconjugated genistein at the 50th percentile would be 8.9–26.7 ng/ml (based on total genistein of 891 ng/ml) and at the 95th percentile the levels would be 27.6–82.9 ng/ml (based on a total genistein of 2763.8 ng/ml). These estimates of unconjugated genistein in infant blood are similar to the estimated levels of unconjugated genistein in the F_1 rats on PND 21 or PND 140 in the NTP multigenerational study at a dietary dose level of 500 ppm where adverse effects were reported (**Table 6**). The estimated levels of genistein (total and estimated aglycone) in human infants are also similar to blood levels measured in mice following oral or sc injection treatment with 50 mg/kg bw/day genistein oral treatment 37.5 mg/kg bw/day genistin on PND 1–5 (**Figure 4 and Figure 5**).

Estimated concentrations of free genistein and daidzein at blood levels corresponding to the 75th percentile for infants fed soy formula are approximately 13.39 ng/ml (0.054 µM) and 1.92 ng/ml (0.008 µM). These estimated values for genistein and daidzein are ~116,000 and ~16,700 times higher, respectively, than an estimated free E2 in serum from infants of 0.000115 ng/ml (see Appendix 1 for the basis of these calculations). The estimated blood levels of free genistein and daidzein in infants overlap with concentrations of these isoflavones predicted to elicit estrogenic activity based on potency estimates relative to estradiol from cell-based transcription assays, which range from 0.000001 to 0.002 (1×10^{-6} to 2×10^{-3}) for genistein and 0.0000024 to 0.00014 (2.4×10^{-6} to 1.4×10^{-4}) for daidzein as summarized by the UK Committee on Toxicity Report on Phytoestrogens and Health (UK Committee on Toxicity 2003)]. Data do not exist to permit a similar comparison based on tissue levels

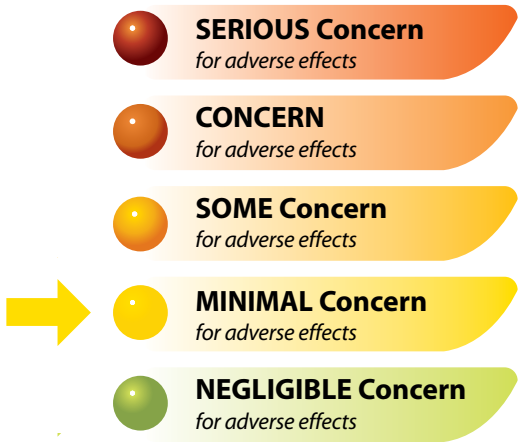
[12] The study by Chang *et al.* (2000) also included measurement of genistein (total and aglycone) in a number of tissues in the rats on PND 140.

of isoflavone in infants. However, genistein and daidzein display relatively weak affinities for serum binding proteins (Nagel *et al.* 1998) and there do not appear to be barriers for tissue uptake (Chang *et al.* 2000) leading to the prediction that tissue concentrations of the aglycones would similarly fall within the range considered estrogenic based on the *in vitro* assays.

The NTP concurs with the conclusion of the CERHR Expert Panel on Soy Infant Formula that there is *minimal concern* for adverse effects on development in infants who consume soy infant formula.

This level of concern represents a "2" on the five-level scale of concern used by the NTP (**Figure 7**). It is based primarily on findings from studies in laboratory animals exposed to genistein, the primary isoflavone in soy infant formula. The existing epidemiological literature on soy infant formula exposure is insufficient to reach a conclusion on whether soy infant formula does or does not cause adverse effects on development in humans. There is "clear evidence" for adverse effects of genistein on reproductive development and function in female rats and mice manifested as accelerated puberty (i.e., decreased age at vaginal opening), abnormal estrous cyclicity, cellular changes to the female reproductive tract, and decreased fecundity (i.e., decreased fertility, implants, and litter size). Also, Infants fed soy infant formula can have blood levels of total genistein that exceed those measured in neonatal or weanling rodents following treatment with genistein at dose levels that induced adverse effects in the animals. However, the NTP accepts the conclusions of the expert panel that the current literature in laboratory animals is limited in its utility for reaching conclusions for infants fed soy infant formula. The NTP agrees with the expert panel that the individual isoflavone studies of genistein, or its glucoside genistin, in laboratory animals would benefit from data on the effects of mixtures of isoflavones and/or other components present in soy infant formula because these mixture studies would better replicate human infant exposures. In addition, a limitation of many of the studies that observed adverse effects in rodents is that exposure occurred during the period of gestation, lactation, and beyond weaning, which made it difficult to distinguish the effects of isoflavones that might have occurred as a result of exposure during lactation alone. A better approximation of human exposure of infants fed soy infant formula would be data from animals exposed during lactation only. Thus, the NTP is initiating a series of studies to address several of the limitations in the laboratory animal studies identified by the expert panel.

Figure 7. NTP Conclusions Regarding the Possibilities that Human Development Might be Adversely Affected by Consumption of Soy Infant Formula



APPENDIX 1: COMPARISON OF ESTIMATED BLOOD LEVELS OF "FREE" GENISTEIN AND DAIDZEIN IN INFANTS FED SOY FORMULA WITH LEVELS OF "FREE" ESTRADIOL

Estimated concentrations of free genistein and daidzein at blood levels corresponding to the 75th percentile for infants fed soy formula are approximately 13.39 ng/ml (0.054 μM) and 1.92 ng/ml (0.008 μM). These estimated values are ~116,000 and ~16,700 times higher than an estimate of free E2 in serum from infants of 0.000115 ng/ml (Table 7). The estimated levels of free genistein and daidzein overlap with concentrations predicted to elicit estrogenic activity based on potency estimates relative to estradiol from cell-based transcription assays, which range from 0.000001 to 0.002 (1×10^{-6} to 2×10^{-3}) for genistein and 0.0000024 to 0.00014 (2.4×10^{-6} t0 1.4×10^{-4}) for daidzein as summarized by the UK Committee on Toxicity Report on Phytoestrogens and Health (UK Committee on Toxicity 2003)].

Basis of calculations

The estimated free concentrations of genistein and daidzein presented above are based on consideration that both the percentage of genistein and daidzein that circulate in the unconjugated state as well as the estimated portion of the unconjugated forms not bound to serum binding proteins, i.e., "free". For infants, 2% of genistein and daidzein was assumed to circulate in the unconjugated form based on data from adults reporting a range of 1 – 3% (Rufer *et al.* 2008; Setchell *et al.* 2001). Unlike endogenous estrogens, neither genistein nor daidzein are considered to bind with particularly high affinity to serum binding proteins although relatively few studies have tried to quantitate these interactions. Nagel *et al*. (1998) used a relative binding affinity-serum modified access (RBA-SMA) assay to calculate effective free fractions of genistein (45.8%), daidzein (18.7%), and equol (49.7)

Table 7. Estimated Circulating Levels of "Free" Estradiol in Infants and "Free" Genistein and Daidzein in Infants Fed Soy Formula

Infant Population	*Blood Concentration, ng/ml (μM)*					
	Estradiol		*Genistein*		*Daidzein*	
	Total	*Free*[1]	*Total*	*Free*[1]	*Total*	*Free*[1]
Females (16d–3y, reference value)[2]	0.013	0.00013				16,695
Males (16d–3y, reference value)[2]	0.010	0.00010				
Average female and male (16d–3y, reference value)	0.0115	0.000115				
Infants fed soy formula, (male and female combined, 75th percentile)[3]			1455.1 (5.4 μM)	13.39 (0.054 μM)	518.7 (2.0 μM)	1.92 (0.008 μM)
Ratio of free isoflavone to free estradiol				116,434 (1.16×10^5)		16,696 (1.67×10^4)

[1] For estradiol (E_2), free concentrations were calculated based on the assumption that ~1% of total E2 is circulating in free form. This value is based on a study by Radfer *et al*. (1976) that 1.03% and 0.75% of E2 is in the free form in 6-week old female and male infants. Free genistein and free daidzein concentrations were estimated at 0.92% and 0.37% (Table 8).

[2] Reference values from Elmlinger *et al*. (2002).

[3] From Cao *et al*. (2009).

Table 8. Estimated Percentage of Genistein and Daidzein Circulating as "Free" (Unconjugated and Unbound to Serum Binding Proteins) in Human Serum

Parameter	*Genistein*	*Daidzein*
Unconjugated [1]	2%	2%
Percent unconjugated that is not bound to serum binding proteins [2]	45.8%	18.7%
Free	0.916%	0.374%

[1] A central estimate based on the range of unconjugated reported at steady state of 1–3% (Rufer *et al.* 2008; Setchell *et al.* 2001).

[2] The "effective free fraction" calculated from relative binding affinity-serum modified access (RBA-SMA) assays by Nagel *et al.* (1998).

in serum from adult human males. The isoflavones were considered to have enhanced access to cells compared to estradiol, which had an effective free fraction of 3.46% based on whole cell uptake saturation assay. If the Nagel *et al*. (1998) values for the isoflavones are combined with a value of 2% present as unconjugated, then an estimated 1% of genistein and 0.4% of daidzein would be present as "free" in circulation, i.e., unconjugated and unbound (Table 8).

The estimated percent of estradiol circulating as free was based on combining data from two publications. One is a 1976 publication by Radfer *et al*. (1976) that reported total and free estradiol levels in human infants during the first weeks after birth (estimates of percent free were not provided in male infants prior to 2 weeks of age). The second publication reported reference levels of total estradiol from infancy to adolescence in a clinically normal set of German children (Elmlinger *et al.* 2002) (Table 9). Radfer *et al*. (1976) reported a sharp decline in levels of total estradiol for both male and female infants during the first weeks of life; however, the percentage free in female infants was constant during this time. The percentage of free estradiol levels decreased from 2.9% at 24-hours to 1.0 % at 6 weeks. In males, the percent free at 6 weeks was lower compared to females, at 0.75% (Table 9). The percent free was similar in boys and girls at 0–8 years of age, 0.81% and 0.87%, respectively. By way of comparison, ~1.8 and 2.3% of total estradiol is free in adult men and women, respectively (Nagel and vom Saal 2004).

Table 9. Reference Values for Serum Concentration of Estradiol in Neonates, Children, and Adolescents

Age	Female			Male			Reference
	Concentration, pmol/l (pg/ml)			Concentration, pmol/l (pg/ml)			
	Total, mean or percentile	Free	n	Total, mean or median	Free	n	
2–6 h	1362 (371), mean	2.84%	8	1358 (370), mean	–	2	Radfar *et al.* (1976)
12 h	349 (95), mean	2.80%	8	224 (61), mean	–	2	
24 h	217 (59), mean	2.90%	8	272 (74), mean	–	2	
72 h	103 (28), mean	2.52%	8	160 (43.5), mean	–	2	
2 w	–	1.48%	8	–	1.01%	3	
6 w	–	1.03%	8	–	0.75%	3	
1–7 d	81(22)*, 50^{th} 25(7)–116(32), 25^{th}–97.5^{th}		17	22(6), 50^{th} <20(<5)–229(62), 25^{th}–97.5^{th}		28	Elmlinger *et al.* (2002)
8–15 d	88(24)*, 50^{th} 42(11)–134(37), 25^{th}–97.5^{th}		20	66(18), 50^{th} 31(8)–126(34), 25^{th}–97.5^{th}		20	
16 d– 3 y	48(13), 50^{th} 21(6)–113(31), 25^{th}–97.5^{th}		44	37(10), 50^{th} <20(<5)–65(18), 25^{th}–97.5^{th}		42	
4–6 y	54(15), 50^{th} <20(<5)–81(22), 25^{th}–97.5^{th}		23	46(13), 50^{th} 29(8)–121(33), 25^{th}–97.5^{th}		28	
7–8 y	59(16), 50^{th} 23(6)–88(24), 25^{th}–97.5^{th}		24	45(12), 50^{th} 20(5)–83(23), 25^{th}–97.5^{th}		26	
9–10 y	47(13), 50^{th} <20(5)–176(48), 25^{th}–97.5^{th}		40	46(13), 50^{th} <20(<5)–81(22), 25^{th}–97.5^{th}		31	
16 d–10y	54(15), 50^{th} 21(6)–109(30), 25^{th}–97.5^{th}		131	44(12), 50^{th} 22(6)–85(23), 25^{th}–97.5^{th}		127	

Table 9 (continued)

Age	Female			Male			Reference
	Concentration, pmol/l (pg/ml)			Concentration, pmol/l (pg/ml)			
	Total, mean or percentile	Free	n	Total, mean or median	Free	n	
11 y	92(25)*, 50th 33(9)–188(51), 25th–97.5th		23	46(13), 50th 28(8)–110(30), 25th–97.5th		22	
12 y	56(15), 50th <20(<5)–221(60), 25th–97.5th		18	45(12), 50th 26(7)–131(36), 25th–97.5th		17	
13 y	79(22)*, 50th <20(<5)–157(43), 25th–97.5th		25	44(12), 50th <20(<5)–232(63), 25th–97.5th		21	
14 y	170(46)*, 50th 42(11)–541(147), 25th–97.5th		30	64(17), 50th 22(6)–273(74), 25th–97.5th		32	
15 y	170(46)*, 50th 25(7)–909(248), 25th–97.5th		48	77(21), 50th <20(<5)–302(82), 25th–97.5th		40	Elmlinger *et al*. (2002)
16 y	230(63)*, 50th 76(21)–849(231), 25th–97.5th		40	83(23), 50th 40(11)–137(37), 25th–97.5th		31	
17 y	163(44)*, 50th 49(13)–507(138), 25th–97.5th		30	58(16), 50th 40(11)–103(28), 25th–97.5th		22	
18–19 y	222(60), 50th 53(14)–688(187), 25th–97.5th		12	52(14), 50th 28(8)–129(35), 25th–97.5th		8	
17–19 y	194(53)*, 50th 51(14)–586(160), 25th–97.5th		42	56(15), 50th 35(10)–109(30), 25th–97.5th		30	

Concentrations presented as pmol/l; conversion factor for pg/ml=(pmol/l value)/3.671
*Significant difference between sexes at the corresponding age

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National Toxicology Program
U.S. Department of Health and Human Services

Center for the Evaluation of Risks to Human Reproduction

APPENDIX I

NTP-CERHR SOY INFANT FORMULA EXPERT PANEL

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APPENDIX I.
NTP-CERHR SOY INFANT FORMULA EXPERT PANEL

A 14-member panel of government and non-government scientists, and was prepared with assistance from NTP staff At a public meeting held on December 16-18, 2009 (74 FR 53509), the expert panel discussed these studies, the adequacy of available data, and identified data needed to improve future assessments. The finalized report presents conclusions on (1) the strength of scientific evidence that soy infant formula or its isoflavone constituents are developmental toxicants based on data from *in vitro*, animal, or human studies; (2) the extent of exposures in infants fed soy infant formula; (3) the assessment of the scientific evidence that adverse developmental health effects may be associated with such exposures; and (4) knowledge gaps that will help establish research and testing priorities to reduce uncertainties and increase confidence in future evaluations. Panel conclusions were based on the scientific evidence available at the time of the public meeting. The NTP-CERHR released the final expert panel report on January 15, 2010 (75 FR 2545. The NTP-CERHR Expert Panel Report on Genistein and Soy Infant Formula is provided in Appendix II. The expert panel report is also available on the CERHR website *(http://ntp.niehs.nih.gov/go/evals)*

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FINAL CERHR EXPERT PANEL REPORT ON SOY INFANT FORMULA

January 15, 2010

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PREFACE

The National Toxicology Program (NTP) and the National Institute of Environmental Health Sciences (NIEHS) established the NTP Center for the Evaluation of Risks to Human Reproduction (CERHR) in June 1998. The purpose of CERHR is to provide timely, unbiased, scientifically sound evaluations of human and experimental evidence for adverse effects on development and reproduction caused by substances to which humans may be exposed. CERHR is headquartered at NIEHS, Research Triangle Park, NC. Additional information about the center and documents related to previous evaluations are available at *http://ntp.niehs.nih.gov/go/ohat*.

Soy infant formula contains soy protein isolates and is fed to infants as a supplement to or replacement for human milk or cow milk. Soy protein isolates contains estrogenic isoflavones ("phytoestrogens") that occur naturally in some legumes, especially soybeans. Phytoestrogens are non-steroidal, estrogenic compounds. In plants, nearly all phytoestrogens are bound to sugar molecules and these phytoestrogen-sugar complexes are not generally considered hormonally active. Phytoestrogens are found in many food products in addition to soy infant formula, especially soy-based foods such as tofu, soy milk, and in some over-the-counter dietary supplements. Soy infant formula was selected for expert panel evaluation because of (1) the availability of large number of developmental toxicity studies in laboratory animals exposed to the isoflavones found in soy infant formula (namely, genistein) or other soy products, as well as a number of studies on human infants fed soy infant formula, (2) the availability of information on exposures in infants fed soy infant formula, and (3) public concern for effects on infant or child development.

The current assessment of soy infant formula is an update to a previous CERHR expert panel evaluation completed in 2006. On March 15–17, 2006, CERHR convened an expert panel to conduct evaluations of the potential developmental and reproductive toxicities of soy infant formula and its predominant isoflavone constituent genistein. The expert panel reports were released for public comment on May 5, 2006 (71 FR 28368). On November 8, 2006 (71 FR 65537), CERHR staff released draft NTP Briefs on Genistein and Soy Formula that provided the NTP's interpretation of the potential for genistein and soy infant formula to cause adverse reproductive and/or developmental effects in exposed humans.

CERHR has not completed these evaluations, finalized the briefs, or issued NTP-CERHR monographs on these substances. Since 2006, a substantial number of new publications related to human exposure or reproductive and/or developmental toxicity have been published for these substances.

CERHR determined that updated evaluations of genistein and soy infant formula were needed. However, the current evaluation focuses only on soy infant formula and the potential developmental toxicity of its major isoflavone components, e.g., genistein, daidzein (and estrogenic metabolite, equol), and glycitein. This updated evaluation does not include an assessment on the potential reproductive toxicity of genistein following exposures during adulthood as was done in the 2006 evaluation. CERHR narrowed the scope of the evaluation because the assessment of reproductive effects of genistein following exposure to adults was not considered relevant to the consideration of soy infant formula use in infants during the 2006 evaluation. To obtain updated information about soy infant formula for the CERHR evaluation, the PubMed (Medline) database was searched from February 2006 to August 2009 with genistein/genistin, daidzein/daidzin, glycitein/glycitin, equol, soy, and other relevant keywords. References were also identified from the bibliographies of published literature.

The updated draft expert panel report represents the efforts of a 14-member panel of government and non-government scientists and was prepared with assistance from NTP staff. The CERHR expert panel used the draft Expert Panel Report on Soy Infant Formula to reach conclusions on (1) the strength of scientific evidence that soy infant formula or its isoflavone constituents are developmental toxicants based on data from *in vitro*, animal, or human studies; (2) the extent of exposures in infants fed soy infant formula; (3) the assessment of the scientific evidence that adverse developmental health effects may be associated with such exposures; and (4) knowledge gaps that will help establish research and testing priorities to reduce uncertainties and increase confidence in future evaluations. The expert panel reached conclusions for soy infant formula at a public meeting held December 16-18, 2009. This final report has been reviewed by members of the soy infant formula expert panel and by CERHR staff scientists. Copies have been provided to the NTP Executive Committee, which is made up of representatives of NTP-participating government agencies. An abbreviated version of the final Expert Panel report was published in Birth Defects Research Part B [Volume 92, Issue 5, October 2011, Pages: 421–468].

The Expert Panel report on Soy Infant Formula was considered extensively by NTP staff in preparing the 2010 NTP Brief on Soy Infant Formula, which represents the NTP's opinion on the potential for exposure to soy infant formula to cause adverse developmental effects in humans. The NTP concurred with the expert panel that there is *minimal concern* for adverse effects on development in infants who consume soy infant formula. This conclusion was based on information about soy infant formula provided in the expert panel report, public comments received during the course of the expert panel evaluation, additional scientific information made available since the expert panel meeting, and peer reviewer critiques of the draft NTP Brief by the NTP Board of Scientific Counselors on May 10, 2010[1]. The Board voted in favor of the minimal concern conclusion with 7 yes votes, 3 no votes, and 0 abstentions. One member thought the conclusion should be *negligible* concern and 2 members thought the level of concern should be higher than *minimal* concern. The NTP's response to the May 10, 2010 review ("peer-review report") is available on the NTP website at *http://ntp.niehs.nih.gov/go/9741.* This monograph includes the NTP Brief on Soy Infant Formula as well as the final Expert Panel report on Soy Infant Formula. Public comments received as part of the NTP's evaluation of soy infant formula and other background materials are available at *http://ntp.niehs.nih.gov/go/36476*.

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[1] Meeting materials are available at *http://ntp.niehs.nih.gov/go/9741*.

NOTES TO READER

The findings and conclusions presented in the final expert panel report are those of the expert panel and should not be interpreted as representing the views of the NTP.

This report is prepared according to the Guidelines for CERHR Panel Members established by NTP/NIEHS. The guidelines are available on the CERHR web site (*http://ntp.niehs.nih.gov/go/ohat*). The format for Expert Panel Reports includes synopses of studies reviewed, followed by an evaluation of the Strengths/Weaknesses and Utility (Adequacy) of the study for CERHR evaluation. Statements and conclusions made under Strengths/Weaknesses and Utility evaluations are those of the expert panel and are prepared according to the NTP/NIEHS guidelines. In addition, the expert panel often makes comments or notes limitations in the synopses of the study. Bold, square brackets are used to enclose such statements. As discussed in the guidelines, square brackets are used to enclose key items of information not provided in a publication, limitations noted in the study, conclusions that differ from those of the authors, and conversions or analyses of data conducted by the expert panel.

This document is not completely 508 compliant. If you have difficulty accessing this document, contact Dr. Kristina Thayer at *http://ntp.niehs.nih.gov/go/ohat* or 919-541-5021.

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ABBREVIATIONS

ANCOVA	analysis of covariance
ANOVA	analysis of variance
AUC	area under the time-concentration curve
BLQ	below the limit of quantification
BMD_{10}	benchmark dose, 10% effect level
$BMD_{1\ SD}$	benchmark dose, 1 control standard deviation
BMDL	benchmark dose 95th percentile lower confidence limit
bw	body weight
C_{max}	maximum plasma concentration
CASA	computer-assisted sperm analysis
CERHR	Center for the Evaluation of Risks to Human Reproduction
CI	confidence interval
CYP	cytochrome P450
DNA	deoxyribonucleic acid
ELISA	enzyme-linked immunosorbent assay
EPA	Environmental Protection Agency
ER	estrogen receptor(s)
FDA	Food and Drug Administration
FSH	follicle-stimulating hormone
g	gram(s)
GC	gas chromatography
GD	gestation day(s)
GLP	good laboratory practice
GnRH	gonadotropin-releasing hormone
HDL	high-density lipoprotein
HPLC	high performance liquid chromatography
IFL	isoflavone
ip	intraperitoneal(ly)
IQ	intelligence quotient(s)
IU	international unit
UIER	urinary isoflavone excretion rate
iv	intravenous(ly)
kcal	kilocalorie(s)
kD	kilodalton(s)
kg	kilogram(s)
kJ	kilojoule(s)
LC	liquid chromatography
LDL	low-density lipoprotein
LH	luteinizing hormone
M	molar
mEq	milliequivalent(s)
mg	milligram(s)
mol	mole(s)
mRNA	messenger ribonucleic acid
MS	mass spectrometry
ng	nanogram(s)

NADPH	nicotinamide adenine dinucleotide phosphate
NCI	National Cancer Institute
NICHD	National Institute of Child Health and Human Development
NIEHS	National Institute of Environmental Health Sciences
NIH	National Institutes of Health
nM	nanomolar
NTP	National Toxicology Program
ODMA	*O*-desmethylangolensin
OECD	Organization for Economic Co-operation and Development
OR	odds ratio
oz	ounce(s)
P450s	cytochrome P450
PAS	periodic acid-Schiff
PCR	polymerase chain reaction
PCNA	proliferating cell nuclear antigen
pg	picogram(s)
pM	picomolar
PND	postnatal day(s)
ppm	parts per million
r^2	coefficient of determination
R_s	Spearman's rank coefficient of correlation
RIA	radioimmunoassay
RM3, RM1	Rat and Mouse No. 3, Rat and Mouse No. 1
RNA	ribonucleic acid
RR	risk ratio
RT	reverse transcription
sc	subcutaneous(ly)
SD	standard deviation
SDN-POA	sexually dimorphic nucleus of the pre-optic area
SEM	standard error of the mean
siRNA	small interfering RNA, silencing RNA
SULT	sulfotransferase
t_{max}	time to maximum plasma concentration
TPO	thyroid peroxidase
UDPGT	uridine diphosphate-glucuronosyltransferase
UGT	UDP-glucuronosyltransferases, uridine 5'-diphospho-glucuronosyltransferase
USDA	US Department of Agriculture
V_d/F	bioavailability fraction
WISC-R	Wechsler Intelligence Scale for Children—Revised
µg	microgram(s)
µL	microliter(s)
µM	micromolar

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1.0 CHEMISTRY, USE, AND HUMAN EXPOSURE

1.1 Chemistry

1.1.1 Nomenclature

The terms "soy," "soybean," "soya," and "soya bean" are commonly used for the leguminous Asian plant, species Glycine max. Soybean is also used to designate the edible seed of this plant. In this report, the term "soy" is used as an adjective to denote products derived from the edible seed (e.g., soy milk, soy infant formula, soy meal) and soybean is used to refer to the edible seed itself.

Soy isoflavones such as genistein, daidzein, and glycitein exist in several forms, as aglyones which have no sugar residue attached or as three glucoside conjugates, beta-, acetyl-, and malonyl-glucosides which have a sugar group attached to position 7 of the A ring *(1)*. Carbohydrate conjugates are generically called glycosides and glucose conjugates are called glucosides. The terms genistein, daidzein, and glycitein refer specifically to the unconjugated aglycones. Isoflavone glucosides are the major isoflavone glycosides and are called genistin, daidzin, and glycitin. Others are acetyl- and malonyl- glycosides (Figure 1). The term "total" genistein, daidzein, or glycitein is used in this report to refer to the respective aglycone and its conjugates.

The Chemical Abstracts Service (CAS) registry number for genistein is 446-72-0 and the International Union of Pure and Applied Chemistry (IUPAC) name is 5,7-dihydroxy-3-(4-hydroxyphenyl)-chromen-4-one. Synonyms for genistein include 4',5,7-trihydroxyisoflavone, 5,7,4'-trihydroxyisoflavone, genisterin, prunetol, and sophoricol. The CAS registry number of daidzein is 486-66-8 and the IUPAC name is 7-hydroxy-3-(4-hydroxyphenyl)-chromen-4-one. Synonyms include 4',7-dihydroxyisoflavone, 7,4'-dihydroxyisoflavone, and daidzeol. For glycitein, the CAS registry number is 40957-83-3 and the IUPAC name is 7-hydroxy-3-(4-hydroxyphenyl)-6-methoxy-chromen-4-one. Synonyms include 7,4'-dihydroxy-6-methoxyisoflavone and 7-hydroxy-3-(4-hydroxyphenyl)-6-methoxy-4H-1-benzopyran-4-one *(2)*.

1.1.2 Formula and Molecular Mass of Genistein, Daidzein, and Glycitein

The molecular formulas, molecular masses, and structures for genistein, daidzein, glycitein and their derivatives are listed in **Figure 1** *(2-4)*.

Figure 1. Chemical Structures of Isoflavones Found in Soy Formula

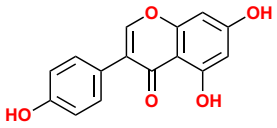
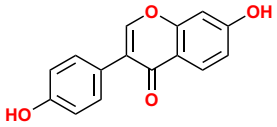
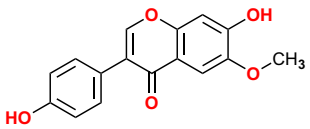
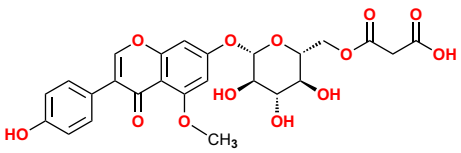
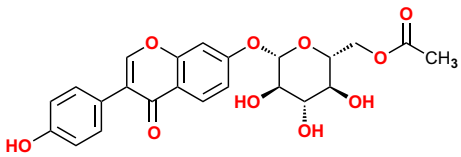
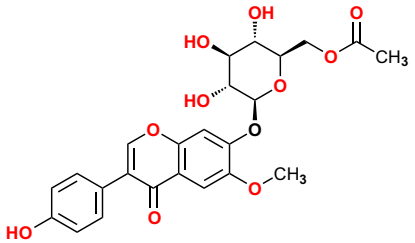
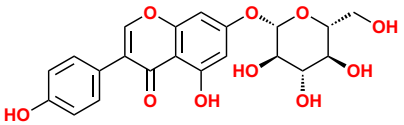
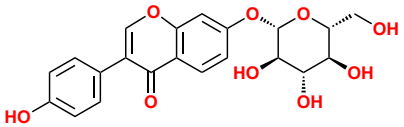
Isoflavone	*Structure*	*IUPAC Name*
Genistein C15H10O5 MW: 270.24 CASRN: 446–72–0		5,7-dihydroxy-3-(4-hydroxyphenyl)-chromen-4-one
Daidzein C15H10O4 MW: 254.24 CASRN: 486–66–8		7-hydroxy-3-(4-hydroxyphenyl) chromen-4-one
Glycitein C16H12O5 MW: 284.26 CASRN: 40957–83–3		7-hydroxy-3-(4-hydroxyphenyl)-6-methoxychromen-4-one

Figure 1 (continued)

Isoflavone	*Structure*	*IUPAC Name*
Acetylgenistin C23H22O11 MW: 474.41 CASRN: 73566–30–0		*2 isomers:* [(2R,3S,4S,5R,6S)-3,4,5-trihydroxy-6-[5-hydroxy-3-(4-hydroxyphenyl)-4-oxochromen-7-yl]oxyoxan-2-yl]methyl acetate] [(2S,3R,4R,5S,6R)-3,4,5-trihydroxy-6-[5-hydroxy-3-(4-hydroxyphenyl)-4-oxochromen-7-yl]oxyoxan-2-yl]methyl acetate
Acetyldaidzin C23H22O10 MW: 458.41 CASRN: 71385–83–6		[(2R,3S,4S,5R,6S)-3,4,5-trihydroxy-6-[3-(4-hydroxyphenyl)-4-oxochromen-7-yl]oxyoxan-2-yl]methyl acetate
Acetylglycitin C24H24O11 MW: 488.44 CASRN: 134859–96–4		[(2R,3S,4S,5R,6S)-3,4,5-trihydroxy-6-[3-(4-hydroxyphenyl)-6-methoxy-4-oxo-chromen-7-yl]oxy-tetrahydropyran-2-yl] methyl acetate
Genistin C21H20O10 MW: 432.37 CASRN: 529–59–9		5-hydroxy-3-(4-hydroxyphenyl)-7-[(2S,3R,4S,5S,6R)-3,4,5-trihydroxy-6-(hydroxymethyl) oxan-2-yl]oxychromen-4-one
Daidzin C21H20O9 MW: 416.37 CASRN: 552–66–9		3-(4-hydroxyphenyl)-7-[(2S,3R,4S,5R,6R)-3,4,5-trihydroxy-6-(hydroxymet hyl) oxan-2-yl]oxy-chromen-4-one

1.1.3 Composition of Soy Infant Formula

Soy infant formula refers to infant food made using soy protein isolate and other components. The term, "soy formula" is often used as a synonym for "soy infant formula" in this report. Soy products contain genistin, genistein and other phytoestrogens of the isoflavone class *(3; 5; 6)*. The 3 main isoflavones found in soy formula are genistein, daidzein, and to a smaller extent, glycitein.

The genistein content of soy formula has been the subject of commentary because of concern about exposure of infants to estrogenic compounds; however, soy formula and other soy-based foods contain many components of which genistein is only one. Soy formula may also contain non-isoflavone constituents of toxicological interest. For example, scientists at Health Canada reported detecting melamine in liquid and powdered infant formula, including soy-based formulas *(7)*, attributed to it being a degradation product of a legally-used insectide. However, in all instances, the concentrations of melamine detected were below the health standard set by Health Canada.

In this report, conclusions regarding soy formula are primarily based on consideration of the literature for

early-in-life exposures to genistein, daidzein (and equol its estrogenic metabolite), glycitein, isoflavone mixtures, soy formulas and other soy-based products to humans or experimental animals. The literature on effects resulting from developmental exposure is much more extensive for genistein, soy diets, or isoflavone mixtures compared to daidzein, equol, or glycitein. Isoflavones found in soy formula are also metabolized to a number of other compounds, such as *O*-desmethylangolensin (ODMA) from daidzein; however these compounds are essentially uncharacterized with respect to potential developmental toxicity.

In the US, the nutrient composition of soy and other infant formulas is regulated by the Food and Drug Administration (FDA) and must comply with the Infant Formula Act of 1980 and subsequent amendments passed in 1986 *(8)*. The specified nutrient levels are based on the recommendations of the Committee on Nutrition of the American Academy of Pediatrics and are reviewed periodically as new information becomes available. **Table 1** lists the primary ingredients in some common brands of powdered soy infant formulas. Those ingredients include corn syrup, soy protein isolate, vegetable oils, soy oil, mortierella alpina oil (source of ARA), crypthecodinium cohnii oil (source of DHA) and sugar *(9)*. In addition, the formulas are fortified with nutrients such as iron, calcium, phosphorous, magnesium, zinc, manganese, copper, iodine, selenium sodium, potassium, chloride, choline, inositol and vitamins A, C, D, E, K, and B (B1, B2, B6, B12, niacin, folic acid, pantothenic acid, biotin). The typical reconstitution of powdered formula is the addition of 8.7–9.3 g powdered formula to 2 fluid ounces of water (1*0*). Soy formulas are also available as concentrated liquids and as ready-to-feed formulations *(11)*.

Table 1. Primary Ingredients in Powdered Enfamil, Isomil Brands and Nestle Good Start Soy Formulas

Ingredient	*Percentage [weight assumed]*
Corn syrup or other source of carbohydrate such as corn maltodextrin	42.6 – 58.0
Soy protein isolate	14.0 – 17.0
Vegetable oils	20.0 – 29.0
Sugar [a]	10.1 – 10.3

[a] Listed as sucrose in some formulations but not specified in others.
From *http://www.drugstore.com (9)*, and Gerber < *http://www.gerber.com/products/*>. See nutrition information for Nestle® GOOD START Soy PLUS™ 2 Formula.

Contaminants of soy protein include phytates (1.5%), which bind minerals and niacin, and protease inhibitors, which have antitrypsin, antichymotrypsin, and antielastin properties (*12*). As discussed further in **Section 1.2.1** formulas are fortified with minerals to compensate for phytate binding and heated to inactivate protease inhibitors. Aluminum is found in soy formulas at concentrations of 600–1300 ng/mL, levels that exceed aluminum concentrations in human milk, 4–65 ng/mL (*12*). Mineral salts added to soy formulas are the source of aluminum.

1.1.4 Chemical and Physical Properties of Genistein and Other Isoflavones Found in Soy Products

In unfermented soy products, small amounts of genistein and other isoflavones (daidzein and to a lesser extent glycitein) are present as aglycones, the unconjugated forms. Most genistein and other isoflavones in unfermented soy products are conjugated to a sugar molecule to form glycosides. Chen and Rogan (*13*) reported that only 3.2–5.8% of total isoflavone in soy formula consists of unconjugated genistein

and daidzein, and that amounts can vary by batch. The majority of isoflavones detected in soy formula are conjugated to sugar molecules to form glycosides *(6)*. Glucose in glycosides can be esterified with acetyl or malonyl groups to form acetyl- or malonylglycosides *(3)*. Heat treatment can degrade the malonyl glucosides into acetyl glucosides, glycosides, and/or aglycones, depending on conditions. Long-term storage at ambient temperature has been reported to decrease the malonyl glucosides and increase glycosides and aglycones (reviewed in Mortensen *et al.*, 2009 (*14*)). Genistein derivatives were the most abundant isoflavones found in 11 varieties of soybeans *(3)*.

As a result of bacterial hydrolysis during fermentation, aglycones represent a larger portion of isoflavones in miso, tempeh, and soybean paste *(3; 15)*. Isoflavones in cooked soybeans, texturized vegetable protein, and soy milk powder are more than 95% glycosides. Tofu, made from precipitated soy milk curd, contains isoflavones with ~20% as aglycones, and tempeh, a fermented soybean product, ~40% aglycones (reviewed in Xu *et al.*, 2000 *(16)*). **Table 2** compares genistein and genistin levels in some unfermented and fermented soy foods (reviewed in ILSI, 1999 *(15)*).

Table 2. Genistein and Genistin Levels in Unfermented and Fermented Soy Foods

Soy Food	*Level, µg/g [mg/100 g]*	
	Genistein	*Genistin*
Soybeans, soy nuts, and soy powder	4.6–18.2 **[0.46–1.82]**	200.6–968.1 **[20.06–96.81]**
Soy milk and tofu	1.9–13.9 **[0.19–1.39]**	94.8–137.7 **[9.48–13.77]**
Miso or natto (fermented)	38.5–229.1 **[3.85–22.91]**	71.1–492.8 **[7.11–49.28]**

From the International Life Sciences Institute (ILSI) *(15)*.

Conjugation with glucose groups increases water solubility of genistein and other isoflavones, which are low molecular-weight hydrophobic compounds *(3)*. Glucoside compounds are deconjugated by gut microflora to form the active aglycone compound *(5)* under acidic conditions *(3)* or by metabolic enzymes *(6)*. Therefore, exposure to a particular isoflavone (e.g., genistein) is theoretically the sum of the aglycone and respective glycoside compound concentrations converted on the basis of molecular weight *(17)*. For example, 1 unit of genistin is equal to 0.6 aglycone equivalents, based on the molecular mass ratio of genistein to genistin (270.24/432.38). However, the aglycone is reconjugated in the gut wall leaving approximately 1–2% free aglycone to enter the portal circulation. Chen and Rogan *(13)* report that isoflavones are glucuronidated and circulate primarily in conjugated form.

1.2 Use and Human Exposure

1.2.1 Production Information

Genistein and other isoflavones are naturally occurring products that can be extracted from soy and other beans. No information on production volume for isoflavones was located. The US was the largest non-Asian producer of soybeans in 2002/2003, averaging 75.11 million metric tons and was forecasted to increase to 80.69 million metric tons by 2005 (reviewed by Choi and Rhee, 2006 *(18)*).

The total isoflavone content of raw, mature soy beans can vary significantly, ranging from 18 to 562 mg/100g *(14)*. Major natural sources of variability in isoflavone content are the cultivated variety of

soybean used and the crop year at the time of cultivation *(14)*. Isoflavone levels can vary as much 2- to 3-fold in different strains grown under similar conditions *(3)*. Other factors that affect isoflavone content in soybeans include geographic location, sowing date, climate, and growing conditions such as temperature and carbon dioxide levels *(6; 14)*.

Specific manufacturing processes of soy foods or soy ingredients can influence the isoflavone content. As reviewed in Choi *et al.*, 2006 *(18)* the following manufacturing steps result in loss of isoflavone content; soaking (12% loss), heat processing (49%) in tempeh production **[attributed to leaching into cooking water]**, coagulation (44%) in tofu processing, and alkaline extraction (53%) in soy protein isolate production. While boiling reportedly reduces genistein content, baking and frying do not appear to alter isoflavone levels in foods *(3)*. Other factors that can affect isoflavone composition in soybean products include use of elicitors during the growth phase *(19)*, pH conditions during processing *(20)*, and choice of starter organism used during the fermentation process *(21-23)*.

With the exception of alcohol extraction, the processing of soybeans does not usually reduce isoflavone content *(15)*. Commercial processing of soybeans can result in decarboxylation, deacetylation, or deglycosylation of glycosides. For example, high temperatures can lead to decomposition of malonyl compounds to their respective acetylglycoside compounds *(3; 6)*. As discussed in **Section 1.1.4,** fermentation leads to a higher percentage of isoflavones as aglycones rather than glycosides *(3)*.

In the manufacture of soy infant formula, the hull of the soybean is removed and the pulp is processed into oil and flake *(12)*. Soy protein isolate is extracted from the flake using a slightly alkaline solution and is precipitated at the isoelectric point of 4.5. The resulting isolate has a purity of ≥90% soy protein. The soy protein isolate is fortified with L-methionine, L-carnitine, and taurine. L-methionine improves the biological quality of the protein. Carnitine is needed for oxidation of long-chain fatty acids. Taurine is a major conjugate of bile acids in infants. Both carnitine and taurine are added at concentrations found in human milk. Vegetable oils such as soy, palm, sunflower, olein, safflower, and coconut are added to provide fats. Corn starch, tapioca starch, and sucrose are used as carbohydrate sources. Phytates, which bind divalent minerals such as calcium, magnesium, iron, and zinc, are present in soy proteins at 1.5%. Therefore, total phosphorus and calcium are added at concentrations that are 20% higher than in cow-milk formulas, and the formulas are supplemented with iron and zinc. Heat applied during the processing of soy protein removes 80–90% of protease inhibitor activity.

1.2.2 Use and Sales of Soy Products and Soy Formula

Exposure to isoflavones occur principally through foods and dietary supplements made with soybeans and soy protein but not soy oils. Other plant parts used as food that have been shown to contain isoflavones include barley (*Hordeum* species) meal, sunflower (*Helianthus*) seed, clover (*Trifolium* species) seed, caraway (*Cuminum cymicum*) seed, peanut (*Arachis hypogaea*), kidney bean (*Phaseolus vulgaris*), chickpea (*Cicer arietinum*), pea (*Pisum sativum*), lentil (*Lens culinaris*), kudzu (*Pueraria lobata*) leaf and root, mungo (*Vigna mungo*) sprout, alfalfa (*Medicago* species) sprout, broccoli (*Brassica oleracea italica*), and cauliflower (*Brassica oleracea botrytis*) *(24)*. Nearly all human isoflavones exposure is attributable to ingestion of soy products. In countries such as India and South American, the traditional diet includes relatively high intakes of kidney beans, lima beans, broad beans, butter beans, chick peas, and/or lentils that can also be important non-soy sources of exposure to isoflavone.

Some of the most common types of soy foods are tofu, soy milk, soy flour, textured soy protein, tempeh, and miso *(25)*. Soy protein can be used in baked goods, breakfast cereals, pasta, beverages, toppings, meat, poultry, fish products, and imitation dairy products such as imitation milk and cheese *(26)*. Soy is present in 60% of processed foods **[not otherwise defined]** available from UK supermarkets *(3)*. The percentage of processed foods containing soy in the US is not known. Exposure to genistein and other isoflavones can also occur through soy supplements marketed for the relief of menopausal symptoms or other purported health benefits.

Based on sales of soy products, it appears that exposure to genistein and other isoflavones in the US is increasing and will continue to increase. The Soyfoods Association of America reports that between 1992 to 2008, sale of soy foods have increased from $300 million to over $4 billion, with a notable increase in sales occurring after the 1999 FDA approval of a health claim linking soy with heart disease reduction *(27)*. Food manufacturers in the U.S. introduced over 2,700 new foods with soy as an ingredient from 2000 to 2007, including 471 new products introduced in 2006 alone. Between 2007 and 2008, the fastest growing markets are for tofu (4.9%), soy milk (3.3%), meat alternatives (8.3%), energy bars (3.1%), and soy cheese, yogurt and ice cream (2.3%). A 2009 report on consumer attitudes sponsored by the United Soybean Board reports that 84 percent of consumers perceive soy products as healthy and that 32% of Americans consume soy foods or soy beverages once a month or more *(28)*. Increases in soy product sales have been attributed to greater knowledge about and interest in longevity and good health by baby boomers, growth of the Asian population in the US, greater intake of Asian foods by Americans, and increased consumption of plant-based foods by young people (reviewed in *(25)*).

A number of studies have reported on the use of soy foods in the context of infant feeding and feeding transitions during the first years of life. Recent data from the Infant Feeding Practices Study II (IFPS II), a longitudinal mail survey of mothers of infants conducted by the FDA in 2005–2007, found that ~6% of infants consume soy foods by 1 year of age (**Table 3**) *(29)*. Infants may also be exposed to soy flour and soy oil by the use of soy-containing fortified spreads (FS) as a complementary food to address growth and nutritional issues in certain countries *(30; 31)*.

Table 3. Percentage of Infants Fed Breast milk, Infant Formula, or Soy Foods in the Previvous 7 Days

Soy Product	*Age, weeks (months)*									
	3–6 (1)	*7–10 (2)*	*11–14 (3)*	*15–18 (4)*	*19–23 (5)*	*24–28 (6)*	*29–35 (7.5)*	*36–42 (9)*	*43–50 (10.5)*	*51–59 (12)*
Number	1961	2240	2302	2101	2139	2046	1990	1920	1779	1782
Breast milk	74.0%	65.4%	61.0%	57.2%	54.6%	50.1%	45.9%	41.7%	36.8%	25.9%
Infant formula	57.2%	61.1%	60.5%	62.9%	64.7%	67.3%	68.9%	70.8%	70.9%	36.4%
Soy foods	0.0%	0.0%	0.1%	0.2%	0.3%	0.7%	1.2%	2.7%	3.7%	5.8%

From Grummer-Strawn et al., 2008 *(29)*.

While the IFPS II indicated that ~57 to 71% of infants were fed infant formula during the first 10 months of life, many aspects of formula use patterns are uncharacterized. For example, it is unknown what percent of infants are exclusively fed formula compared to what percent are fed a mixture of infant formula and breast milk. It is also unknown what proportion of formula-fed infants are exclusively fed soy formula. A 1998 infant-feeding survey conducted by Ross Products Division indicated that 18% of infants are fed

soy formula during the first year of life *(32)*. That percentage of use, when combined with data from the 2000 US census of 4 million American infants younger than 1 year of age, provided the basis for Strom *et al.*, 2001 *(32)* to estimate that 750,000 US infants per year are fed soy formula. Another estimate was that 25% of newborns in the US are fed soy formula *(33)*. A study conducted at Yale University examined formula changes in 189 breast-fed infants and 184 formula-fed infants and reported that a total of 87 infants **[23%]** received soy formula sometime during the first 4 months of life *(34)*.

Market sales data are often cited as a surrogate measure for soy formula usage. According to the International Formula Council (IFC), the international trade association for manufacturers and marketers of infant formula, 12% of US infant formula dollar sales between June-September 2009 were from soy-based formula (personal communication with Robert Rankin, Manager of Regulatory and Technical Affairs at the IFC, October 13, 2009). This value is based on consumer spending data from Nielsen[1], which represents about 60% of the total US market including the Women, Infants, and Children (WIC) program. Formula sales data from Wal*Mart, club stores (e.g., Costco and BJ's), and Babies R Us/ Toys R Us are not provided to Nielsen; however, the majority of formula sales by these stores are milk-based routine formulas. The 12% of sales value compiled for June-September 2009 is representative of longer periods of recent sales. Dollar sales of soy formula were 11.6% for the 52 week period ending on September 5, 2009. Other types of formula, categorized as "Routine milk-based formula" and "Specialty or Tolerance Formulas" represented 61.2% and 27.2% of dollar sales, respectively (personal communication with Jeremy Jones, Director of Sales Strategy for PBM Products, LLC on October 14, 2009). In the US between 1999 and 2009, estimates of total soy infant formula fed decreased from 22.5% to 12.7% calculated based on total formula sold corrected for differences in formula cost. i.e., expressed in equivalent feeding units (public comment from the International Formula Council (IFC), received December 3, 2009 and personal communication with Dr. Haley Curtis Stevens, IFC).

These recent sales data, when assumed to be representative of actual usage provide a lower estimate of soy formula usage when compared to actual usage reporting .

The usage and sales of soy formula vary geographically ranging from 2 to 7% of infant formula sales in the UK, Italy, and France , 13% in New Zealand, and 10–25% in the US *(35; 36)*. A telephone survey in Israel identified soy formula feeding in 31.5% of 1803 infants at some time during the first year of life *(37)*. Of the children on soy formula, 65% used it for 12 months or more. The decision to use soy formula as opposed to cow-milk formula was made by the mother rather than a health care provider in the majority of instances in the Israeli survey. The mother's decision was most often based on her personal preference rather than concerns for cow milk allergies or for symptoms.

Other commonly cited reasons for use of soy formula are to feed infants who are allergic to dairy products or are intolerant of lactose, galactose, or cow-milk protein *(38; 39)*. Infants are often changed from cow-milk to soy formula when they have symptoms such as colic, crying, diarrhea, and vomiting *(34)*. Some parents feed their infants soy formula to maintain a vegetarian lifestyle or because of perceived health benefits of soy food consumption *(33)*. Soy formula is not currently recommended for preterm infants. Manufacturers of soy formula and some brand names of soy formula sold in the US are listed in **Table 4**.

[1] Nielsen is a major marketing and media information company that measures product sales, market share, distribution, price, and merchandising conditions in retail outlets such as grocery stores, drug stores, mass merchandisers and convenience stores.

Table 4. US Soy Formula Manufacturers and Brand Names

Manufacturer	*Brand Names*
Mead Johnson	Enfamil ProSobee LIPIL
Nestle *(Carnation, Gerber)*	Alsoy Good Start Soy DHA & ARA GOOD START Soy PLUS
Ross *(Abbott Nutrition)*	Similac Go and Grow Soy-based Milk Similac Isomil Advance
PBM *(formerly known as Wyeth)*	AAFES/NEXCOM Baby's Choice Soy Infant Formula AAFES/NEXCOM Baby's Choice Soy Infant Formula with DHA & ARA Albertson's Baby Basics Soy Infant Formula with DHA & ARA HyVee Mother's Choice Soy Infant Formula HyVee Mother's Choice Soy Infant Formula with DHA & ARA Kozy Kids Soy-based Infant Formula with DHA & ARA Kroger Comforts Soy Infant Formula with Iron and DHA & ARA Parent's Choice Infant Formula with Soy and DHA & ARA PathMark Soy Infant Formula with DHA & ARA Price Chopper Soy Infant Formula with Iron and DHA & ARA Rite Aid Soy Infant Formula with DHA & ARA Target Soy with Iron Target Soy Infant Formula with Iron and DHA & ARA Top Care Soy Infant Formula with DHA & ARA Walgreens Soy Protein Formula with Iron and DHA & ARA Wegman's Soy Infant Formula with Iron and DHA & ARA Western Family Soy Infant Formula with DHA & ARA
Vermont Organics	Soy Organic Infant Formula
Wyeth-Ayerst	Nursoy

Brand names are registered to their owners. Store brand formulas are also sold in the US and the manufacturers may be othert than those listed above. From *(40; 41)*.

1.2.2.1 Guidelines on Use of Soy Formula

In May 2008, the American Academy of Pediatrics (AAP) released an updated policy statement on the use of soy protein-based formulas *(42)*. The overall conclusion of the AAP was that, although isolated soy protein-based formulas may be used to provide nutrition for normal growth and development in term infants, there are few indications for their use in place of cow milk-based formula. The only real indications for use are incidences where the family prefers a vegetarian diet or for the management of infants with galactosemia or primary lactase deficiency (rare). Similar overall conclusions on the use of soy-based formula were reached in other recent reviews and evaluations *(35; 36; 43; 44)*.

Specific conclusions in the 2008 AAP report include:

- Lactose free and reduced lactose-containing cow milk formulas are now available and could be used for circumstances in which elimination or a reduction in lactose in the diet, respectively, is required. Because primary or congenital lactase deficiency is rare, very few individuals would require a total restriction of lactose. Lactose intolerance is more likely to be dose dependent. Thus, the use of soy protein-based lactose-free formulas for this indication should be restricted.

- The routine use of isolated soy protein-based formula has no proven value in the prevention or management of infantile colic or fussiness.
- Isolated soy protein-based formula has no advantage over cow milk protein-based formula as a supplement for the breastfed infant, unless the infant has one of the indications noted above.
- Soy protein-based formulas are not designed for or recommended for preterm infants. Serum phosphorus concentrations are lower, and alkaline phosphatase concentrations are higher in preterm infants fed soy protein-based formula than they are in preterm infants fed cow milk-based formula. As anticipated from these observations, the degree of osteopenia is increased in infants with low birth weight receiving soy protein-based formulas. The cow milk protein-based formulas designed for preterm infants are clearly superior to soy protein-based formula for preterm infants.
- For infants with documented cow milk protein allergy, extensively hydrolyzed protein formula should be considered, because 10% to 14% of these infants will also have a soy protein allergy.
- Infants with documented cow milk protein-induced enteropathy or enterocolitis frequently are as sensitive to soy protein and should not be given isolated soy protein-based formula. They should be provided formula derived from hydrolyzed protein or synthetic amino acids.
- The routine use of isolated soy protein-based formula has no proven value in the prevention of atopic disease **[hypersensitivity reactions, allergic hypersensitivity affecting parts of the body not in direct contact with the allergen]** in healthy or high-risk infants.

In 2006, the European Society for Paediatric Gastroenterology Hepatology and Nutrition (ESPGHAN) Committee on Nutrition published a medical position paper on soy protein infant formula and follow-on formula *(36)*. Their seven primary conclusions on the use of soy protein-based infant formula were:

- Cow's milk-based formula should be preferred as the first choice for feeding healthy infants that are not fully breast fed.
- Soy protein based formula should only be used in specified circumstances because they may have nutritional disadvantages and contain high concentrations of phytate, aluminum, and phytoestrogens, the long-term effects of which are unknown.
- Indications for soy formula include severe persistent lactose intolerance, galactosemia, religious, ethical, or other considerations that stipulate the avoidance of cow's milk based formulae and treatment of some cases of cow's milk protein allergy.
- The Committee recommends that the use of therapeutic formulae based on extensively hydrolyzed proteins (or amino acid preparations if hydrolysates are not tolerated) should be preferred to that of soy protein formula in the treatment of cow's milk protein allergy. Soy protein formula should not be used in infants with food allergy during the first 6 months of life. If soy protein formulae are considered for therapeutic use after the age of 6 months because of their lower cost and better acceptance, tolerance to soy protein should first be established by clinical challenge.
- Soy protein formulae have no role in the prevention of allergic diseases.
- There is no evidence supporting the use of soy protein formula for the prevention or management of infantile colic, regurgitation, or prolonged crying.
- Manufacturers should aim to reduce the concentrations of trypsin inhibitors, lectins, goitrogenic substances, phytate, aluminum, and phytoestrogens in soy protein formula.

1.2.2.2 Occurrence and Exposure

Analysis of isoflavones can be divided into a series of steps that involve (1) sample collection, (2) disintegration of plant tissues and homogenization of a sample, (3) extraction and purification of crude extracts, (4) separation and identification of individual substances, and (5) detection and quantification (reviewed in Vacek *et al.*, 2008 *(45-47)*. The stability of isoflavones in a sample can be limited by factors such as light, temperature (higher temperatures promote hydrolysis), pH, and solvent. Ideally, samples should be stored in the dark and frozen to minimize degradation. Each step in sample collection and analysis requires attention as isoflavones may interact with constituents of the matrix and the conjugates may undergo hydrolysis. Isolation methods used to remove the target analytes from complex samples are a key step in quantitative analytical determination of the isoflavones. As reviewed in Vacek *et al.*, 2008 *(45)*, sample extraction and purification techniques that use a combination of several methods tend to be more labor intensive but provide the most quantitative recovery, high selectivity in a small extract volume, and precision. These can include solid phase extraction (SPE), accelerated-solvent extraction, and Soxhelt extraction. For example, the combination of sonication, to assist in removing the analyte from the matrix to the extraction solvent, and SPE can result in more concentrated extracts that allow the determination of very low concentrations of isoflavones, especially when a strong and effective solvent is used to free the analyte from the sample matrix. Other easier-to-preform automated methods are also available for sample extractions that are reproducible, but typically less quantitative.

Wang *et al.*, 2002 *(48)* published a review on the analytical methods for isoflavones that included a comparison of the sensitivity, specificity, limits of detection for individual isoflavones, and advantages/disadvantages of the various methods (**Table 5**). The analytical methods for isoflavones could be divided into two general categories: (1) those that have an initial chromatographic separation such as GC and high-performance liquid chromatography (HPLC), and capillary electrophoresis (CE), and (2) those that do not such as matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MAL-DI-TOF-MS), deconvolution UV and infrared (IR) spectroscopy, and immunoassay. HPLC is the most common method used to separate isoflavones used because it requires simple sample preparation, is highly efficient and reproducible, and has been extensively studied *(49)*. Isoflavones in biological samples such as urine, blood, or breast milk are present mostly as glucuronides and sulfates, and sample preparation for these matrices typically includes treatment with b-glucuronidase like that isolated from *Helix pomatia* that contains both glucuronidase and arylsulfatase activities. The growing consumption and promotion of isoflavones as beneficial for health has led to calls to develop internationally harmonized methods for the detection and quantification of isoflavones in food, supplements, and biological samples to address issues related to food laws on safety, contaminants, allowable additive, label claims, and trade agreements (discussed in Akhtar and Abdel-Aal, 2006 *(50)*).

1.2.2.3 Environmental

Phytoestrogens have been detected in aqueous samples from a variety of environmental sources including drainage water from pastures, rivers, creeks and samples from waste water treatment plants using various methods *(51-55)*. There are indications of seasonal patterns in the concentrations detected. For example, in Portugal, levels of genistein and daidzein were maximal in early summer in several river estuaries *(54; 55)*.

Lundgren and Novak, 2009 *(56)* determined the concentrations of phytoestrogens in wastewater streams from 19 plant-processing industries, e.g., biodiesel, ethanol, soy products, peanuts and corn, and three wastewater treatment plants located in the midwestern US. Genistein was detected in 14 of the 19 industry wastewater streams and daidzein was detected in 12 of 19. Concentrations of

Table 5. Analytical Methods for Isoflavones

Technique		*Sensitivity*	*Specificity*	*Pros*	*Cons*
GC-MS		50 fmol	High	• High resolution • Good for unknowns	• Complex work up • Difficult chemistry
HPLC	UV (and DAD)	2 pmol	Moderate Better with DAD	• Good for soy food and conjugates	• Low sensitivity • Less specific
	Fluorescence	200 fmol	Good	• Sensitive	• Limited to fluorescent analytes
	ED (and array)	20 fmol	Better with detection array	• Suitable for biological samples	• Cannot determine novel compounds
	MS	1–500 fmol	High	• Ease of use and sensitive	• Limited chromatographic resolution
CE	UV (and DAD)	50 fmol	Moderate Better with DAD	• High separation resolution • Excellent mass sensitivity	• Limited injection sample volume • Poor concentration sensitivity
	Fluorescence	1–5 fmol	Moderate	• Sensitive	• Limited fluorescent analysis
	ED (and array)	1–2 fmol	Moderate	• Sensitive	• Limited specificity
	MS	100 amol	High	• Sensitive	• Difficult interface • Low resolution
UV and IR Spectroscopy		Not Reported	Fair	• High throughput	• Lack of specificity
MALDI-MS		100 fmol	High	• High throughput	• Lack of quantitation
Immunoassay		1–100 fmol	Good	• High throughput	• Cross reactivity

Modified from Table 1 in Wang et al., 2002 *(48)*.
Abbreviations:
CE=capillary electrophoresis; DAD=diode array detection; ED=electrochemical detection;
GC-MS=gas chromatography-mass spectrometry; HPLC=high performance liquid chromatography;
IR=infrared; MALDI-MS=matrix-assisted laser desorption ionization-mass spectrometry; UV=ultraviolet;

genistein ranged from below the limit of detection to 151,00 ng/L and concentrations of daidzein ranged from below the limit of detection to 108,000 ng/L. The highest concentrations were in effluents from industries processing soy products. Eight of the industries' effluents contained concentrations of total phytoestrogens in excess of 1000 ng/L. Wastewater treatment plants using aerobic biological treatment appeared to effectively remove phytoestrogens.

1.2.2.4 Genistein and Other Isoflavones in Food and Soy Supplements

Food

Intake of soy foods is significantly correlated with urinary genistein and the sum of all isoflavones indicating that nearly all genistein and isoflavones exposure in humans occurs from ingestion of soy products *(57; 58)*. Measurements of the isoflavone content for a wide variety of food items and/or associated dietary intake assessments are available in a number of databases (reviewed in Schwartz *et al.*, 2009 *(59)*). The 17 databases reviewed by Schwartz *et al.*, 2009 *(59)* could be classified as one of three general types, (1) literature compendia or reviews to help identify sources of isoflavone exposure (n=4), (2) dietary intake assessments based on food frequency questionnaires or national food

consumption studies (n=10), and (3) comprehensive databases for nutritional research that provide sufficient information on food samples, analytical methods, and the quality of individual values to be used as a resource in nutrition research by regulatory agencies, food industry, and scientists (n=3). Not all of the databases reviewed in Schwartz *et al.*, 2009 are publically accessible.

One of the major publically-available databases for the US of isoflavone content in food was created by the Agricultural Research Service (ARS) of the USDA and recently updated in September 2008 *(60)*. The "USDA database," formally titled the "USDA Database for the Isoflavone Content of Selected Foods - Release 2", was compiled from an extensive review of various data sources and scientific articles published in peer-reviewed journals. A total of 265 articles were retrieved through literature searches and 66 articles contained acceptable analytical data for isoflavone content of foods and food ingredients. These data were merged with the earlier data from 1999 to update the database *(61)*. All the data were evaluated by a data quality evaluation system developed by the Nutrient Data Laboratory of the ARS/USDA. The 2008 update of the USDA database contains values for genistein, daidzein, and glycitein (expressed as aglycone equivalents) for 557 food items, including food items which may contain soy ingredients. These food items included bakery products like bread, doughnuts, and muffins, meat products like sausages and canned food items like tuna or meatless chili in the production of which soy flour (bakery products) or soy protein (meat products, chili) may have been added. The database also includes isoflavone content for soy- and cow's milk-based infant formula and those data are discussed in **Section 1.2.2.5**.

USDA data on isoflavone content for select foods are presented in (**Table 6**). The data presented in the USDA database for the main food sources of isoflavones are generally consistent with reports and reviews by others. Among soy foods, the highest quantities of isoflavones and their glycosides are found in soybeans and soy flour (*(24)*, reviewed in Mortensen *et al.*, 2009 *(14)*). The main food sources are soy foods, "new generation" soy products such as soy burgers and soy cheese, and commonly consumed foodstuffs in the production of which soy flour or soy protein isolates are used *(14)*. Similar to the data presented in the USDA database, Mazur *et al.*, 1998 reported non-measureable levels of isoflavones in fruits and berries. Cruciferous vegetables such as cauliflower and broccoli had non-detectable to very low levels of isoflavones.

Table 6. Isoflavone Contents in Various Food Items

Database Entry Number	*Food Item*	*Average Content*			
		Genistein (mg/100 g)	*Daidzein (mg/100 g)*	*Total Isoflavone (mg/100 g)*	*Protein (g/100 g)*
Soy Foods and Products					
16115	Soy flour, full fat, raw	99	73	178	34.5
16111	Soybeans, mature seeds, roasted (soy nuts)	76	62	149	39.6
16122	Soy protein isolate	57	31	91	80.7
43212	Bacon, meatless, unprepared	46	64	118	32
08385	Kellogg's SMART START soy protein	42	42	94	
99072	Soybean chips	27	27	54	—
11450	Soybeans, green, ras (includes edamame)	23	20	49	12.9

Table 6 (continued)

Database Entry Number	Food Item	Average Content			
		Genistein, mg/100 g	Daidzein, mg/100 g	Total Isoflavones, mg/100 g	Protein, g/100 g
16112	Miso	23	16	41	11.7
99510	Soy yogurt	17	14	33	—
16427	Tofu, regular, raw	13	9	23	8.1
16114	Tempeh	36	23	61	—
16168	Soy milk, fluid, chocolate, with added calcium, vitamins A and D	4	3	7.8	2.6
16113	Natto	38	33	82	—
99472	Soy cheese American	9	6	18	—
16173	Chicken nuggets, meatless, canned, prepared	9		15	5
16107	Sausage, meatless	9	4	14	18.5
16147	Veggie vor soy burger	5	2	6	15.7
Legumes and Legume Products					
16006	Beans, baked, canned, plain or vegetarian	0.01	0	0.01	t
16014	Beans, black, mature seeds, raw	0	0.01	0.01	—
99503	Black bean sauce	4	6	10	—
16032	Beans, kidney, red, mature seeds, raw	0.01	0.01	0.02	—
16056	Chickpeas (garbonzo beans, bengal gram), mature seeds, raw	0.06	0.23	0.38	—
16085	Peas, split, mature seeds, raw	0.11	0.33	0.44	
Vegetables, Fruits, and Fruit Juices					
11001	Alfalfa seeds, sprouted, raw	0.02	0.02	0.04	
11011	Asparagus, raw	0	0.03	0.03	
99549	Broccoli sprouts, raw	0	0.04	0.04	—
99571	Clover, red	10	11	21	—
09116	Grapefruit, raw, white, all areas	0.03	0.04	0.06	
09209	Orange juice, chilled, includes from concentrate	0.01	0.01	0.01	—
09298	Raisins, seedless	0.05	0.03	0.08	—
11529	Tomatoes, red, ripe, raw, year round avg	0	0	0	—
Dairy, Eggs, Poultry, Meat and Seafood					
05327	Chicken breast tenders, uncooked	0.25	0.20	0.55	—
01123	Egg, whole, raw, fresh	0.02	0.03	0.05	—
99533	Non–dairy creamer, with added soy flour or soy protein	0.14	0.06	0.21	—
99485	Ensure, liquid nutrition	2.58	1.40	4.33	—
15184	Fish, tuna, light, canned in water, without salt, drained solids	0.05	0.04	0.09	—

Table 6. (continued)

Database Entry Number	Food Item	Average Content			
		Genistein (mg/100 g)	Daidzein (mg/100 g)	Total Isoflavones (mg/100 g)	Protein (g/100 g)
15185	Fish, tuna, white, canned in oil, without salt, drained solids	0.15	0.12	0.28	—
07022	Frankfurter, beef	0.80	1.00	1.90	—
23501	USDA Commodity, beef patties with VPP, frozen, cooked	1.09	0.67	1.86	—
Nuts and Seeds					
12061	Nuts, almonds	0.01	0	0.01	—
12087	Nuts, cashew nuts, raw	0.01	0	0.01	—
12151	Nuts, pistachio nuts, raw	1.75	1.88	3.63	—
12220	Seeds, flaxseed	0.04	0.02	0.07	—
Other Beverages					
14006	Alcoholic beverage, beer, light	0	0	0	—
14003	Alcoholic beverage, beer, regular, all	0	0	0	—
14003	Alcoholic beverage, wine, table, red, Merlot	0	0	0	—
14209	Coffee, brewed from grounds, prepared with tap water	0.01	0.03	0.04	—
14201	Coffee, brewed from grounds, prepared with tap water, decaffeinated	0	0	0	—
99107	Tea, green, Japan	0.02	0.01	0.02	—
14355	Tea, brewed, prepared with tap water	0	0	0	—
Baked Products, Fast Food, and Sweets					
18248	Doughnuts, plain	2.44	2.58	5	5.87
18035	Bread, multi-grain (includes whole grain)	0.15	0.20	0.38	—
18069	Bread, white commercially prepared (includes soft bread crumbs)	0.13	0.06	0.19	—
18127	Cake, snack cakes, crème-filled, chocolate with frosting	0.15	0.13	0.20	—
18356	Sweet rolls, cinnamon, commercially prepared with raisins	0.65	0.70	1.50	—
99564	Desserts, frozen, Glace Soy milk	6.2	7.0	14	—
99555	JACK IN THE BOX, Beef Monster Taco	13.1	2.60	15.9	—
22903	Pizza, pepperoni topping, regular crust, frozen, cooked	0.01	0.01	0.01	—
99557	Subway, meatball sandwich	2.7	3.0	6.0	—

Data obtained from the USDA Database for the Isoflavone Content of Selected Foods – Release 2 < *http://www.ars.usda.gov/Services/docs.htm?docid = 6382*> and a presentation prepared by scientists at the Nutrient Data Laboratory of the ARS/USDA (Joanne M. Holden, Seema Bhagwat, and David Haytowitz) on July 28 – 29, 2009 ("Soy Protein/Isoflavone Research: Challenges in Designing and Evaluating Intervention Studies," NIH workshop held in Bethesda, MD on July 28 – 29, 2009)

The isoflavone content of soybeans show considerable variability when based on samples from the US or when all sources are combined (**Table 7**). The review by Schwartz *et al.*, 2009 *(59)* also noted significant variation in isoflavone content for similar food items reported in multiple databases . This observation was not unexpected given all the factors that can influence isoflavone content and measurement such as cultivar, environmental conditions, processing methods, sample work-up, and analytical method. For example, six databases presented information on total isoflavone content of soybeans in a manner that allowed comparison and estimates ranged from 469–2389 mg/kg fresh weight. Total isoflavone values of 362-2209, 1421, and 1036 mg/kg fresh weight were reported in the 1999 version of the USDA database *(11)*, and those developed by Ritchie *et al.*, 2006 *(62)*, and Thompson *et al.*, 2006 *(63)*, respectively.

Table 7. Variability in Isoflavone Content of Soybeans

Source	*Average Isoflavone Content, mg/kg (range)*			
	Genistein	*Daidzein*	*Glycitein*	*Total Isoflavones*
U.S.	86 (20–180)	61 (10–191)	13 (1–122)	160 (18–388)
All sources	81 (6–276)	62 (3–191)	15 (10–122)	155 (10–440)

From a presentation prepared by scientists at the Nutrient Data Laboratory of the ARS/USDA (Joanne M. Holden, Seema Bhagwat, and David Haytowitz) on July 28–29, 2009 ("Soy Protein/Isoflavone Research: Challenges in Designing and Evaluating Intervention Studies," NIH workshop held in Bethesda, MD on July 28–29, 2009).

Other food items of interest were not reported in the USDA database but have been discussed elsewhere. For example, Setchell *et al.*, 1998 reported detecting only trace levels of isoflavones in soy oil *(64)*. A review by Mazur *et al.*, 1998 *(24)* indicated that Kudzu root, used as an herbal medication and, to a lesser extent as a food, contained genistein and its glycoside at 12.6 mg/100 g dry weight. Kuhnle *et al.*, 2008 *(65)* analyzed the phytoestrogen content (isoflavones: biochanin A, daidzein, formononetin, genistein, and glycitein; lignans: secoisolariciresinol and matairesinol; coumestrol; equol; enterolactone; and enterodiol) of 115 foods of animal origin including dairy products, eggs, meat, fish, and seafood as well as vegetarian substitutes. Isoflavones were detected in all foods of animal-origin analyzed, although at much lower levels than in soy-based food products (< 10 µg/100 g of wet weight). By way of comparison, the isoflavone content of beef products was 1-3 µg/100 g wet weight and for the meat substitute soy-based/vegetarian burger the isoflavone content was 4410-4415 µg/100 g wet weight.

In addition to the USDA database, two other large, publically accessible databases used for isoflavone intake assessment have been recently published and were included in the review by Schwartz *et al.*, 2009 *(59)*. Thompson and other researchers at the University of Toronto and at Cancer Care, also located in Toronto, developed a database to estimate isoflavone intake for epidemiological and clinical studies *(63)*. Thompson *et al.*, 2006 *(63)* simultaneously measured nine phytoestrogens (including isoflavones, lignans, and coumestan) in 121 food items relevant to Western diets to provide more accurate intake estimations for epidemiological and clinical studies. The relative ranking of phytoestrogen content (based on total analyte measured per 100 g food item) presented from highest to lowest, was nuts and oilseeds, soy products, cereals and breads, legumes, meat products, and other processed foods that may contain soy, vegetables, fruits, alcoholic, and nonalcoholic beverages. Another comprehensive database to estimate isoflavone intake was developed by researchers in the United Kingdom *(62)*. Ritchie *et al.*, 2006 *(62)* measured total genistein and daidzein content in

approximately 300 food items available in the United Kingdom and combined this information with recipe calculations to develop isoflavone content for ~6000 foods.

Haytowitz *et al.*, 2009 *(66)* estimated dietary intake of total isoflavones and contribution by individual food items in the U.S. diet by combining data from (1) the 2008 USDA Database for the Isoflavone Content of Selected Foods, Release 2.0, (2) the 2006 USDA Food and Nutrient Database for Dietary Surveys (FNDDS),[2] and (3) weighted two-day food consumption data for the U.S. population from What We Eat in America (WWEIA)[3], dietary intake component of the National Health and Nutrition Examination Survey (NHANES). Data on isoflavone content for 290 of the 557 foods described in the USDA database was combined with data from 2006 version of the FNDDS to estimate isoflavone content in typical food portions. Then, the dietary intake data from the 2005-2006 WWEIA-NHANES survey was included in the analysis to estimate the dietary intake of total isoflavones and contribution by individual food items. Soy products, such as soymilk, meatless vegetarian products, and tofu are the major contributors of isoflavones to the diet. Other food items, where soy products are used as ingredients (such as doughnuts, frankfurters, infant formulas, bread and rolls) may also contribute significant amounts of isoflavones to the diet (**Table 8**).

The USDA data on relative contribution by individual food items to total isoflavone intake are similar to findings from other Western countries. Ritchie *et al.*, 2006 *(62)* used their database and a 7-day food diary to estimate isoflavone intake in groups of vegetarians (n=10) and omnivores (n=9). In the vegetarian group, the mean isoflavone intake was 7.4 mg/d and main food sources of isoflavones were soya milk (plain), meat-substitute foods containing textured vegetable protein and soya protein isolate, soya mince, wholemeal bread and rolls, white bread and rolls, croissants and pitta breads, beans, raisins and soya sauce. Main food sources of isoflavones for the omnivorous group (mean intake of 1.2 mg/d) were soya yogurts, wholemeal bread and rolls, white bread and rolls, garlic bread, nan bread and brown bread, sultanas and scones.

French *et al.*, 2007 *(67)* conducted a study in premenopausal Canadian women to examine the association between dietary intake of phytoestrogens estimated from food frequency questionnaire with urinary metabolites. **[The degree of correlation between urinary isoflavone concentrations and estimates of dietary intake ranged from 0.5 to 0.6; these data are discussed more in Section 1.2.2.7]**.

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[2] The FNDDS is a database of foods, their nutrient values, and weights for typical food portions. U.S. Department of Agriculture (USDA), Agricultural Research Service. (2008). USDA Food and Nutrient Database for Dietary Studies, 3.0. Food Surveys Research Group Web site: *http://www.ars.usda.gov/Services/docs.htm?docid=17031.*

[3] What We Eat in America (WWEIA), NHANES is a national food survey conducted as a partnership between the U.S. Department of Health and Human Services (DHHS) and the U.S. Department of Agriculture (USDA). WWEIA represents the integration of two nationwide surveys - USDA's Continuing Survey of Food Intakes by Individuals (CSFII) and HHS' NHANES. Under the integrated framework, DHHS is responsible for the sample design and data collection. USDA is responsible for the survey's dietary data collection methodology, development and maintenance of the food and nutrient databases used to code and process the data, and data review and processing. The two surveys were integrated in 2002. U.S. DHHS, National Center for Health Statistics. 2008. National Health and Nutrition Examination Survey 2005-2006 Data Files. *http://www.cdc.gov/nchs/about/major/nhanes/nhanes05-06.htm*

Table 8. Isoflavone Intake Contributed by Key Foods

Food Description	Average Isoflavone Content (range)					
	Total Isoflavones		Genistein		Daidzein	
	Content, mg/100 g	% of Total Isoflavone Intake	Content, mg/100 g	% of Total Genistein Intake	Content, mg/100 g	% of Total Daidzein Intake
Soy milk (all types and flavors)	7.8	22.3	4.2	24.5	3.4	21.5
Tofu (all kinds)	24.1	12.3	12.9	13.6	9.6	11.2
Chicken	0.53	12.3	0.24	11.6	0.19	9.9
Meatless products (includes veggie burgers and other imitation vegetarian products)	24.2	12.1	11.3	11.7	11.6	12.6
Soybeans	65.1	5.2	31.3	5.2	30.8	5.5
Coffee	0.03	5.1	0.01	3.0	0.03	9.1
Doughnuts	1.37	5.1	0.6	4.7	0.7	5.6
Frankfurters (various meats)	0.60	4.4	0.3	3.8	0.3	5.0
Chicken, fried (nuggets, fingers)	0.55	2.8	0.3	3.8	0.2	3.3
Bread and rolls, white	0.10	2.3	0.05	2.9	0.06	2.0
Turkey	0.5	2.2	0.2	2.0	0.2	1.6
Infant formula, soy-based	11.8	1.7	5.9	1.8	3.9	1.2
Infant formula, milk-based	0.8	0.5	0.4	0.5	-	-
Sausage (various meats)	0.2	1.2	0.1	1.4	0.1	0.9
Soy sauce	1.2	1.1	0.4	0.7	0.8	1.6
Miso	41.5	1.1	23.2	1.2	16.4	0.9
Formulated bar, POWER BAR, chocolate	5.1	1.0	3.3	1.3	1.8	0.9
Eggs	0.05	0.7	0.02	0.54	0.03	0.9
Peanut butter	0.3	0.7	0.1	0.5	0.2	0.9
Sweet potato	0.3	0.6	0.1	0.6	0.1	0.8

From Haytowitz, D.B., Bhagwat, S.A. 2009. Assessment of sources and dietary intake of isoflavone in the U.S. Diet. Federation of American Societies for Experimental Biology Conference, April 18–22, 2009, New Orleans, Louisiana *(66)*.

The top ten contributors to isoflavone intake were soy beverages (49.6%), tofu (16.2%), soy nuts (10.8%), cooked soy beans (9.8%), soy "meat" alternatives (3.8%), split green peas (2.2%), soy flour (1.9%), miso soup (1.7%), textured vegetable protein or TVP (1.4%), and tofu "yogurt" (1.1%). A similar study by Tseng *et al.*, 2008 *(68)* was conducted in a sample of US women to evaluate the use of a soy food questionnaire and the Willett food frequency questionnaire. Although there was some variation in the top sources of isoflavones compared to the study by French *et al.*, 2007 *(67)*, soy milk, soy nuts, tofu, and soy bars were ranked the highest.

Some information on soy food consumption is available for infants and toddlers. Recent data from the IFPS II indicates that ~6% of infants consume soy foods by 1 year of age (**Table 3**). Gerber sponsors the Feeding Infants and Toddlers Study (FITS), a survey of the eating habits and nutrient intakes of > 3,000 children from 4 to 24 months of age. Based on survey data collected in 2002, Skinner *et al.*, 2004 *(69)* reported soy milk as one of the frequently consumed beverages in children 15-18 months of age, but not in younger infants or toddlers 19-24 months of age. The exact frequency of consumption was not reported but products were not considered frequently consumed unless consumption occurred in > 10% of the sample. A 2006 presentation from the Executive Director of the Soyfoods Association of North America, Nancy Chapman[4], cited FITS data to say that out of 600 toddlers 15-24 months old, almost 4% consumed soymilk at least once a day. Overall, soy milk is one of the fastest growing markets in the soy food industry *(28)*. However, it is unclear whether this trend extends to infants and toddlers although data obtained from a 2008 update to FITS[5] should be informative.

On September 12, 2008, the USDA Food and Nutrition Service (FNS) published a final rule in the Federal Register (73 FR 52903), that provides detailed nutritional standards for nondairy alternatives to milk in federally subsidized school lunches, breakfasts or after-school snacks. This rule allows school nutrition programs to serve nondairy beverage alternatives to fluid milk, including soy milk, as long as certain nutritional standards are met in the product and a written substitution request is provided by the parent or legal guardian. Schools were also granted the discretion to select acceptable nondairy beverages and given the option of providing milk substitutes to children with milk allergies, religious or ethical beliefs or other needs that preclude the consumption of milk but are not based on a medical disability. Some common brands of soymilk sold in the U.S. include WestSoy, EdenSoy, Silk Soymilk, Wildwood SOYmilk, and ZenSoy *(70)*.

Although phytoestrogens are more typically associated with soy products, equol, a metabolite of daidzein, is routinely measured in cow's milk. Cows are considered to "equol producers" whereas less than half of humans readily produce this estrogenic metabolite. Leguminous plants used in cattle nutrition, such as red clover and alfalfa, contain high concentrations of phytoestrogens, resulting in significant equol production in the cows that graze on these forage plants. King *et al.*, 1998 *(71)* conducted HPLC analyses on Australian cow milk and reported mean isoflavone levels of <5 ng/mL daidzein, 4–29 ng/mL genistein, and 45–293 ng/mL equol. The amount of equol detected in cow's milk

[4] Presentation available at *http://www.soyfoods.org/wp/wp-content/uploads/2006/12/soymilk_in_school_meals.pdf.*

[5] Nestlé Nutrition is funding Mathematica to conduct the 2008 Feeding Infants and Toddlers Study (FITS 2008), a followup to the FITS 2002 study. This survey will obtain updated information on the diets and eating habits of U.S. infants and toddlers 0 to 48 months of age. The time frame for the project is 2008-2009 (*http://www.mathematica-mpr.com/nutrition/fits2008.asp)*.

varies and appears to vary based forage plant selection and growing season. Equol concentrations may be higher in organic milk products presumably because organic dairy cows eat more forage legumes compared to conventionally raised cows. The forage legumes bind nitrogen from the air thereby making them a tool in reducing fertilizer use. For example, Hoikkala *et al.*, 2007 *(72)* reported ~6.5 times higher concentrations of equol in Finnish samples of organic cow's milk than in conventional milk (411 ng/mL compared to 62 ng/mL). In Finland, the legume red clover is commonly used in organic farms whereas barley, oats, and rapeseed meal are used on conventional dairy farms. U.S. retail sales of organic milk have been growing since the mid-1990s, with sales of organic milk and cream up 25 percent from 2004. In contrast, overall sales of milk have remained constant since the mid-1980s and organic milk and cream now make up an estimated 6 percent of retail milk sales *(73)*.

Dietary supplements

Exposure to genistein and other isoflavones can occur through intake of dietary supplements often used because of the perception that they can improve cardiovascular health or reduce the symptoms of menopause (recent reviews on the use of soy or isoflavone supplements for health benefit are summarized in Chapter 2. When compared to soy food products, including soy-based infant formula, the highest isoflavone concentrations are found in nutritional supplements which may be consist of up to 40% total isoflavones (reviewed in Mortensen *et al.*, 2009 *(14)*).

A large number of publications have reported on the isoflavone content of botanicals and soy supplements. The NTP-CERHR Expert Panel Report on the Reproductive and Developmental Toxicity of Genistein and Soy Formula is not intended to provide a comprehensive presentation of this literature. However, it is clear that isoflavone content in dietary supplements is quite variable and presents a challenge to designing and evaluating intervention studies of soy protein or isoflavones. A further challenge is that the reporting in this literature is often imprecise in describing whether the measured value represents the aglycone or the combination of aglycone and glycoside. In addition to the reports described below, a number of other studies have been published since the 2006 NTP-CERHR evaluation of genistein and soy formula that report isoflavone content in dietary supplements *(74; 75)*.

Setchell *et al.*, 2001 *(76)* analyzed 33 commercially available phytoestrogen supplements to determine the types and levels of compounds present. **[Either the information provided by the author or the types of compounds identified in the supplements indicated that 28 of the supplements were derived from soybeans.]** The composition of the supplements was highly variable, and many contained unidentified compounds. The soy-based supplements consisted primarily of genistein, daidzein, and glycitein-derived glycosides. Aglycones represented <10% of the formulation for the majority of soy-based supplements (22/28). Five of the soy-based supplements contained 10–26% aglycones, and 1 of the supplements contained 47.2% aglycones. Total isoflavones per capsule or serving were measured at 2.8–58.0 mg for the soy-based supplements. Isoflavone levels were found to vary by more than 10% of the manufacturers' reported values for about half of the 33 phytoestrogen supplements analyzed. The UK Committee on Toxicity *(3)* reported that 4 surveys of soy supplements found that actual levels of isoflavones differed from values listed on labels and that, in most cases, actual levels were below those reported by manufacturers.

Doerge *et al.*, 2000 *(77)* measured isoflavone levels in a soy supplement purchased at a local health food store. The majority of isoflavones were present as acetyl glucosides and malonyl glucosides.

Total genistein content (aglycone + conjugates) was 4 mg/tablet and total daidzein (aglycone + conjugates) was 8.9 mg/tablet. The values represented 84% of daidzein levels and 48% of genistein levels listed on the product label.

More recently, Grippo *et al.*, 2007 *(78)* measured concentrations of 5 flavonoids, including genistein and daidzein, in 19 botanical and ephedra-containing dietary supplements. Over half (11/19) of the supplements contained measurable amounts of genistein and the majority of the supplements (17/19) contained measurable amounts of daidzein, with a total isoflavone concentration of up to 22 mg/day per recommended dosage. The amount of genistein and daidzein in the supplements expressed as mg per recommended daily dose (RDD) ranged from 0.0330 to 7.30 mg/RDD for genistein to 0.024 to 17.7 mg/RDD for daidzein.

Thompson *et al.*, 2007 *(79)* evaluated 21 non-vitamin and non-mineral dietary supplements for phytoestrogen analysis. Brands that were analyzed were the most frequently reported non-vitamin and non-mineral supplements used in a random survey of 479 Canadian women conducted in 2003. This study reports total isoflavone concentration values within the range of other studies evaluating supplements. In addition, as reported in other studies there seems to be discrepancies in the label claim and the analytical data presented in this study. Study data indicated amounts per tablet/capsule were 2-3 times lower than the stated amounts on the product label. The supplement with the highest weights of genistein, daidzein, and glycitein contained 9814.1 mg/tablet of genistein, 3130.5 mg/tablet of daidzein and 363 mg/tablet of glycitein. Because the recommended label dose is for two capsules per day, use of this supplement may result in a daily dose of ~19,600 mg/day of genistein, ~6260 mg/day of daidzein and ~725 mg/day of glycitein.

In 2005, the Agency for Healthcare Research and Quality, part of the US Department of Health and Human Services, released a report it had commissioned entitled "Effects of Soy on Health Outcomes" to assess the clinical trial literature on soy supplements and soy foods *(80)*. Of the soy supplement trials, 57 % used soy protein with isoflavones, 36 % used isoflavones alone, and 6 % used soy protein without isoflavones. The total isoflavone intake in the reviewed studies ranged from 0 to 185 mg per day and the total protein intake from soy ranged from 0 g to 154 g per day. The median intake of soy protein per day, 36 g, was considered equivalent to over 1 pound of tofu or ~ 3 soy protein shakes per day. For individual isoflavones, the range of intakes based on ingestion of a supplement or soy food was 0−85.2 mg/day for genistein, 0−75 mg/day for daidzein, and 0−66 mg/day for glycitein.

1.2.2.5 Genistein and Other Isoflavone Levels in Infant Formula and "Weaning" Foods

Levels of isoflavones infant soy formula are summarized in **Table 9**. This table includes information reported in the survey of soy foods conducted by the US Department of Agriculture (USDA) and Iowa State University *(11)* as well as other sources. Unpublished data and analyses conducted at Iowa State University are included in the USDA-IOWA survey. Methodological details on most of the studies cited in **Table 9** are briefly summarized below. Results are presented for the most common isoflavones, genistein, daidzein, and glycitein, although some studies did not include glycitein values. Glycoside values were converted to free-form (aglycone) values. Total isoflavones were calculated if values were available for daidzein and genistein equivalents, but it was noted that reported total isoflavone values may not equal values obtained by adding individual isoflavone equivalents. The cited literature includes results for formula powders, liquid concentrates, and reconstituted or ready-to-eat formulas. As expected, levels of isoflavone equivalents expressed as mg/100 g were higher in soy formula powders

and liquid concentrates. Percentages for individual isoflavones were genistein equivalents 36.8–70.1%, daidzein equivalents 18.2–45.8%, and glycitein equivalents 4.0–14.8%. [To aid in making comparisons, CERHR converted the measured values to a common metric of "ready to feed (mg/L)" using assumptions described in the annotations below **Table 9**. Reconstituted or ready-to-feed soy formulas purchased in the US contained genistein equivalents 13.6–~32.4 mg/L formula *(6; 81)*, daidzein equivalents 7.5–19.1 mg/L formula (Murphy *et al.*, unpublished data as cited in USDA-Iowa Database *(11)*; *(6)*, glycitein equivalents 2.6–4.2 mg/L formula (*(81)*; Murphy *et al.*, 1997 and unpublished data as cited in USDA-Iowa Database *(11)*) and total isoflavone equivalents of 20.9–47 mg/L formula *(6; 81)*. Estimated isoflavone intakes for soy formula fed infants based on these summary values are presented in **Table 26**.

The UK Ministry of Agriculture, Fisheries, and Food summarized and compared levels of isoflavones and their conjugates measured in soy formula from other countries, and concluded that isoflavone levels in soy formula from the UK were similar to concentrations reported in other countries; differences most likely resulted from batch variations in soy isolate isoflavone levels, slight variations in formulas, and minor differences in analytic methodology. A comparison of levels of isoflavones in formula normalized to "as prepared" from different countries is presented in **Table 10**. The lowest total isoflavone content of 10 mg/L was measured in a sample of Nestle Alsoy powdered formula purchased in São Paulo by Genovese *et al.*, 2002 *(88)* and the highest, 47 mg/L, was reported for a sample of Abbott Nutrition (formerly Ross), by Setchell *et al.*, 1998 *(6)*.[6]

[6] A higher isoflavone content of 93 mg/L, based on the sum of genistein and daidzein, was presented for liquid Korean soy infant formula by Lee *et al.*, 2003 [93] **[4.67 mg/100 ml genistein and 4.64 mg/100 ml of daidzein, equal to 46.7 mg/L genistein and 46.4 mg/L daidzein for a combined isoflavone content of 93 mg/L]**. The study also measured the isoflavone content in samples of casein-based formula and breastmilk. In addition, the authors reported urine and plasma concentrations in infants fed soy formula (n=10), casein-based formula (n=14), or breast milk (n=15) for 4 months. Infants in the 3 groups were also compared on weight, length, head circumference, chest circumference, and infant development based on the developmental quotient. However, the main text of the article is written in Korean and many specifics of the study are unknown. Moreover, the isoflavone levels presented in the publication raise concerns on the reporting quality of the study. The value of 93 mg/L in soy formula is approximately twice the amount of the next highest reported level, 47 mg/L by Setchell *et al.*, 1998 [6]; a difference that also represents the typical dilution factor for liquid concentrates which raises uncertainty on whether the liquid formula sample was a "ready-to-feed" sample or a liquid concentrate. **[A visiting scientist at the NIEHS translated the article for CERHR but it did contain additional information on the brand name, type of sample, or number of samples tested]**. Lee *et al.*, 2003 also presented information on serum and urine isoflavone levels in soy formula fed infants, but the presented values are difficult to reconcile with other estimates as the blood-based estimates are lower (plasma=392.1 ng/ml verus 684 from Setchell *et al.*, [84] and 757 from Cao *et al.*, 2009 [94], but the urine values much higher (urine=17.89 µg/ml [17,900 ng/ml] verus 5891 ng/ml from Cao *et al.*, 2009 [94]. Additional concerns are raised about the study because the reported concentrations of genistein + daidzein in breast milk were much higher, 0.16 mg/100ml **[1.6 mg/L, equal to 1600 µg/L or ng/ml]** than any other reported value including a total isoflavone level of 32 ng/ml from breast milk samples collected from vegan women as described in Mortensen *et al.*, 2009 [14]. Yet, despite the much higher reported breast milk levels, the plasma levels of genistein and daidzein in the breastfed infants in the Lee *et al.*, 2003 study, 3.8 and 3.4 ng/ml, were similar to the whole blood levels reported in Cao *et al.*, 2009 [94] (10.8 ng/ml of genistein, 5.3 ng/ml of daidzein) and plasma by Setchell *et al.*, 1997 [84] (2.8 ng/ml of genistein and 1.5 ng/ml of daidzein). CERHR attempted to contact the corresponding author but was not able to obtain clarification on the study. Because of the uncertainty in the published values, the Lee *et al.*, 2003 [93] is not considered in the Expert Panel's evaluation of soy formula.

Table 9. Isoflavone Content in Soy Infant Formulas

Formula Description	Isoflavones in Aglycone Equivalents								References[a]
	Genistein		Daidzein		Glycitein		Total Isoflavone		
	mg/100 g	Ready To Feed mg/L (% total)	mg/100 g	Ready To Feed mg/L (% total)	mg/100 g	Ready To Feed mg/L (% total)	mg/100 g	Ready To Feed mg/L	
US									
ENFAMIL NEXT STEP, powder, soy formula, not reconstituted **[USDA nutrient database number: 03931]**	14.8	**[19.9]**[b,c] (59.0%)[b]	7.2	**[9.8]**[b,c] (28.7%)[b]	3.0	**[4.0]**[b,c] (12.0%)[b]	25.0	**[33.7]**[c]	As cited in the 2008 USDA database *(60)*: Murphy et al., 1997 *(82)*; Murphy, unpublished data
MEAD JOHNSON, GERBER, powder, soy formula with iron, not reconstituted **[USDA nutrient database number: 03863]**	13.9	**[18.8]**[c] (55.4%)[b]	8.1	**[10.9]**[c] (32.2%)[b]	3.1	**[4.2]**[c] (12.5%)[b]	25.1	**[33.9]**[c]	As cited in the 2002 USDA database *(11)*: Murphy et al., 1997 *(82)*; Murphy, unpublished data
MEAD JOHNSON, GERBERSOY, powder, not reconstituted	**[14.9]**[d]	**[20.1]**[c] (57.1%)[b]	**[9.3]**[d]	**[12.5]**[c] (35.6%)[b]	**[1.9]**[d]	**[2.6]**[c] (7.3%)[b]	**[26.1]**[d]	**[35.2]**[c]	Franke et al., 1998 *(81)*
MEAD JOHNSON, PROSOBEE, liquid concentrate, reconstituted	**[~5.78]**[g]	**[~28.9]**[e,f] (~64.3%)	**[~2.66]**[g]	**[~13.3]**[e,f] (~29.5%)	**[(~0.56)]**[g]	**[~2.8]**[e,f] (~6.2%)	**[9.1]**[g]	45	Setchell et al., 1998 *(6)*
MEAD JOHNSON, PROSOBEE with iron, liquid concentrate, not reconstituted **[USDA nutrient database number: 03824]**	2.2	**[11]**[g]	1.1	**[5.5]**[g]	–	–	6	**[30]**[g]	As cited in the 2002 USDA database *(11)*: *Genistein, daidzein:* Nguyenle et al., 1995 *(83)* *Total isoflavones:* Nguyenle et al., 1995 *(83)* Setchell et al., 1997 *(84)*
MEAD JOHNSON, PROSOBEE with iron, powder, not reconstituted **[USDA nutrient database number: 03826]**	14.9	**[20.1]**[c] (59.8%)[b]	7.1	**[9.6]**[c] (28.5%)[b]	3.0	**[3.9]**[c] (11.6%)[b]	24.9	**[33.6]**[c]	As cited in the 2002 USDA database *(11)*: Murphy et al., 1997 *(82)*; Murphy, unpublished data
MEAD JOHNSON, PROSOBEE with iron, powder, not reconstituted	**[15.6]**[d]	**[21.0]**[c] (55.3%)	**[10.2]**[d]	**[13.8]**[c] (36.1%)	**[2.4]**[d]	**[3.2]**[c] (8.5%)	**[28.1]**[d]	**[38.0]**[c]	Franke et al., 1998 *(81)*

Table 9. (continued)

Formula Description	Isoflavones in Aglycone Equivalents								References[a]
	Genistein		Daidzein		Glycitein		Total Isoflavone		
	mg/100 g	Ready To Feed mg/L (% total)	mg/100 g	Ready To Feed mg/L (% total)	mg/100 g	Ready To Feed mg/L (% total)	mg/100 g	Ready To Feed mg/L	
MEAD JOHNSON, PROSOBEE with iron, ready-to-feed **[USDA nutrient database number: 03823]**	2.18	**[21.8]**[d] (56%)	1.71	**[17.1]**[d] (44%)	–	–	3.89	**[38.9]**[d]	Setchell et al., 1987 *(85)* as cited in the 2002 USDA database *(11)*
NESTLE, ALSOY liquid concentrate, reconstituted		**[~20.3]**[e] (~63.4%)		**[~8.6]**[e] (~27%)		**[~3.1]**[e] (~9.6%)		32	Setchell et al., 1998 (6)
NESTLE, ALSOY powder, not reconstituted	**[9.5]**[d]	**[12.8]**[c] (56.7%)	**[5.9]**[d]	**[8.0]**[c] (35%)	**[1.4]**[d]	**[1.9]**[c] (8.3%)	**[16.8]**[d]	**[22.7]**[c]	Franke et al., 1998 *(81)*
ABBOTT NUTRITION (formerly ROSS), SIMILAC, ISOMIL, with iron, powder, not reconstituted **[USDA nutrient database number: 03843]**	12.23	**[16.5]**[c]	6.03	**[8.1]**[c]	2.73	**[3.7]**[c]	25.82	**[34.9]**[c]	As cited in the 2008 USDA database *(60)*: *Genistein, daidzein, glycitein:* Murphy et al., 1997 *(82)*; Murphy, unpublished data; *Total isoflavones:* Murphy et al., 1997 *(82)*; Murphy, unpublished data; Setchell et al., 1997 *(84)*
ABBOTT NUTRITION (formerly ROSS), SIMILAC, with iron, powder, not reconstituted	8.95	**[12.1]**[c] (57.7%)	5.27	**[7.1]**[c] (34%)	1.28	**[1.7]**[c] (8.3%)	15.5	**[20.9]**[c]	Franke et al., 1998 *(81)*
ABBOTT NUTRITION (formerly ROSS), SIMILAC, powder, reconstituted as fed		**[~31.2]**[e,f] (~66.3%)		**[~13.5]**[e,f] (~28.8%)		**[~2.4]**[e,f] (~5%)		47	Setchell et al., 1998 *(6)*
ABBOTT NUTRITION (formerly ROSS), SIMILAC, ISOMIL, with iron, reconstituted from powder, as fed **[USDA nutrient database number: 99112]**	1.58	**[15.8]**[d] (58.3%)	0.78	**[7.8]**[d] (28.8%)	0.35	**[3.5]**[d] (12.9%)	2.71	**[27.1]**[d]	International Formula Council unpublished data (2001) as cited in the 2002 USDA database *(11)*

Table 9. (continued)

Formula Description	Isoflavones in Aglycone Equivalents								References[a]
	Genistein		Daidzein		Glycitein		Total Isoflavone		
	mg/100 g	Ready To Feed mg/L (% total)	mg/100 g	Ready To Feed mg/L (% total)	mg/100 g	Ready To Feed mg/L (% total)	mg/100 g	Ready To Feed mg/L	
ABBOTT NUTRITION (formerly ROSS), SIMILAC, ISOMIL, with iron, ready-to-feed **[USDA nutrient database number: 03841, updated in 2008 to include more samples)**	1.37	**[13.7]**[d]	0.73	**[19.1]**[d]	0.12		2.21	**[22.1]**[d]	As cited in the 2008 USDA database *(60)*: Johns et al., 2003 *(86)*, Setchell et al., 1987 *(85)*
ABBOTT NUTRITION (formerly ROSS), SIMILAC, ISOMIL, with iron, ready to feed **[USDA nutrient database number: 03841, data from 2002)**	2.26	**[22.6]**[d] (~54.2%)	1.91	**[19.1]**[d] (~45.8%)			4.17	**[41.7]**[d]	Setchell et al., 1987 *(85)* as cited in the 2002 USDA database *(11)*
ABBOTT NUTRITION (formerly ROSS), SIMILAC, ISOMIL, with iron, ready-to-feed		**[~28.1]**[e] (~63.9%)		**[~13.2]**[e] (~30.1%)		**[~2.6]**[e] (~6%)		44	Setchell et al., 1998 *(6)*
PBM PRODUCTS (formerly WYETH-AYERST), ULTRA BRIGHT BEGINNINGS, liquid concentrate **[USDA nutrient database number: 03891, some samples freeze dried prior to analysis]**	2.69	–	0.98	–	0.35	–	3.81	Not Calculated Some liquid samples were freeze dried prior to analysis	As cited in the 2008 USDA database *(60)*: Murphy et al., 1997 *(82)* Nguyenle et al., 1995 *(83)*
WYETH-AYERST, NURSOY, powder, reconstituted		**[~32.4]**[e,f] (~70.4%)		**[~12.6]**[e,f] (~27.3%)		**[~1.9]**[e,f] (~4.2%)		46	Setchell et al. 1998 (6)

Table 9. (continued)

Formula Description	*Isoflavones in Aglycone Equivalents*								*References*[a]
	Genistein		*Daidzein*		*Glycitein*		*Total Isoflavone*		
	mg/100 g	*Ready To Feed mg/L (% total)*	*mg/100 g*	*Ready To Feed mg/L (% total)*	*mg/100 g*	*Ready To Feed mg/L (% total)*	*mg/100 g*	*Ready To Feed mg/L*	
PBM PRODUCTS (formerly WYETH-AYERST), ULTRA BRIGHT BEGINNINGS with iron, powder, not reconstituted **[USDA nutrient database number: 03893]**	13.6	**[19.4]**[c] (52.1%)[b]	5.7	**[8.2]**[c] (21.9%)[b]	2.1	**[3.0]**[c] (7.9%)[b]	28	**[37.8]**[c]	As cited in the 2008 USDA database *(60)*: *Genistein, daidzein, glycitein:* Murphy et al., 1997 *(82)* *Total isoflavones:* Murphy et al., 1997 *(82)*; Setchell et al., 1997 *(84)*
PBM PRODUCTS (formerly WYETH-AYERST), ULTRA BRIGHT BEGINNINGS, ready-to-feed **[USDA nutrient database number: 03890]**	1.6	**[16.0]**[d] (60.8%)[b]	0.75	**[7.5]**[d] (28.5%)[b]	0.28	**[2.8]**[d] (10.6%)[b]	2.63	**[26.3]**[d]	Murphy et al., unpublished data as cited in the 2008 USDA database *(60)*
UK									
COW & GATE, powder, reconstituted	**[2.46]**[b,d]	**[24.6]**[b] (60.0%)	**[1.44]**[b,d]	**[14.4]**[b] (35.0%)	**[0.16]**[b,d]	**[1.6]**[b] (4.0%)	**[4.1]**[d]	41	MAFF 1998 report *(5)*
H J HEINZ CO LTD, FARLEY's SOYA , powder, reconstituted	**[1.94]**[b,d]	**[19.4]**[b] (57.0%)	**[1.29]**[b,d]	**[12.9]**[b] (38.0%)	**[(0.17)]**[b,d]	**[1.7]**[b] (5.0%)	**[3.4]**[d]	34	
MEAD JOHNSON, PROSOBEE with iron reconstituted	**[1.36]**[b,d]	**[13.6]**[b] (59.0%)	**[0.78]**[b,d]	**[7.8]**[b] (34.0%)	**[0.16]**[b,d]	**[1.6]**[b] (7.0%)	**[2.3]**[d]	23	
MEAD JOHNSON, PROSOBEE with iron, powder, reconstituted	**[2.38]**[b,d]	**[23.8]**[b] (58.0%)	**[1.52]**[b,d]	**[15.2]**[b] (37.0%)	**[0.20]**[b,d]	**[2.0]**[b] (5.0%)	4.1	41	
ROSS, ISOMIL, with iron, powder, reconstituted	**[1.70]**[b,d]	**[17.0]**[b] (55.0%)	**[1.21]**[b,d]	**[12.1]**[b] (39.0%)	**[0.19]**[b,d]	**[1.9]**[b] (6.0%)	3.1	31	

Table 9. (continued)

Formula Description	Isoflavones in Aglycone Equivalents								References[a]
	Genistein		Daidzein		Glycitein		Total Isoflavone		
	mg/100 g	Ready To Feed mg/L (% total)	mg/100 g	Ready To Feed mg/L (% total)	mg/100 g	Ready To Feed mg/L (% total)	mg/100 g	Ready To Feed mg/L	
SMA NUTRITION, SMA WYSOY BATCH B, powder, reconstituted	**[1.97]**[b,d]	**[19.7]**[b] (60.0%)	**[1.08]**[b,d]	**[10.8]**[b] (33.0%)	**[0.25]**[b,d]	**[2.5]**[b] (8.0%)	3.3	33	MAFF 1998 report *(5)*
SMA NUTRITION, SMA WYSOY BATCH F, powder, reconstituted	**[1.04]**[b,d]	**[10.4]**[b] (58.0%)	**[0.67]**[b,d]	**[6.7]**[b] (37.0%)	**[0.09]**[b,d]	**[0.9]**[b] (5.0%)	1.8	18	
SOY-BASED, powder, not reconstituted	23.2	**[31.3]**[c] (67.1%)	9.3	**[12.5]**[c] (26.8%)	2.1	**[2.8]**[c] (6.1%)	34.6	**[46.7]**[c]	Hoey et al., 2004 *(87)*
	14.6	**[19.7]**[c] (57.9%)	6.7	**[9.0]**[c] (26.5%)	3.9	**[5.3]**[c] (15.5%)	25.2	**[34.0]**[c]	
	21.7	**[29.3]**[c] (64.2%)	9.8	**[13.2]**[c] (29%)	2.3	**[3.1]**[c]	33.8	**[45.6]**[c]	
SOY INFANT FORMULA, powder, not reconstituted							19.2	**[25.9]**[c]	Kuhnle et al., 2008 *(65)*
Brazil									
KASDORF APTAMIL SOJA, powder (imported from Holland, purchased in São Paulo, Brazil)	**[7.9]**[b]	**[10.7]**[c] (55.2%)	**[4.3]**[b]	**[5.8]**[c] (30%)	**[2.1]**[b]	**[2.8]**[c] (14.8%)	14.3	**[19.3]**[c]	Genovese et al., 2002 *(88)*
NUTRICIA APTAMIL SOJA, powder (imported from Argentina, purchased in São Paulo, Brazil)	**[8.2]**[b]	**[11.1]**[c] (53.3%)	**[4.7]**[b]	**[6.3]**[c] (30.5%)	**[2.5]**[b]	**[3.4]**[c] (16.2%)	15.4	**[20.8]**[c]	
NESTLE NESTOGENO, powder (purchased in São Paulo, Brazil)	**[8.6]**[b]	**[11.5]**[c] (51.5%)	**[7.0]**[b]	**[9.4]**[c] (42.2%)	**[1.0]**[b]	**[1.3]**[c] (6.3%)	16.6	**[22.3]**[c]	
MILUPA, PREGOMIN, powder (imported from Germany, purchased in São Paulo, Brazil)	**[8.2]**[b]	**[11.1]**[c] (62%)	**[3.9]**[b] (29.6%)	**[5.3]**[c]	**[1.1]**[b]	**[1.5]**[c] (8.4%)	13.3	**[17.8]**[c]	

Table 9. (continued)

Formula Description	*Isoflavones in Aglycone Equivalents*								*References*[a]
	Genistein		*Daidzein*		*Glycitein*		*Total Isoflavone*		
	mg/100 g	*Ready To Feed mg/L (% total)*	*mg/100 g*	*Ready To Feed mg/L (% total)*	*mg/100 g*	*Ready To Feed mg/L (% total)*	*mg/100 g*	*Ready To Feed mg/L*	
MEAD JOHNSON, PROSOBEE, powder (purchased in São Paulo, Brazil)	**[12.0]**[b]	**[16.2]**[c] (59.3%)	**[6.4]**[b]	**[8.6]**[c] (31.7%)	**[1.8]**[b]	**[2.4]**[c] (9%)	20.3	**[27.4]**[c]	Genovese et al., 2002 *(88)*
NESTLE, ALSOY powder (purchased in São Paulo, Brazil)	**[(4.4)]**[b]	**[6.0]**[c] (59.8%)	**[(1.8)]**[b]	**[2.4]**[c] (23.9%)	**[(1.2)]**[b]	**[1.6]**[c] (16.3%)	7.4	**[10.0]**[c]	
WYETH-AYERST, NURSOY, powder (purchased in São Paulo, Brazil)	**[(6.8)]**[b]	**[9.2]**[c] (57.4%)	**[(3.6)]**[b]	**[4.9]**[c] (30.9%)	**[(1.4)]**[b]	**[1.9]**[c] (11.7%)	11.8	**[15.9]**[c]	
Australia and New Zealand									
INFASOY, reconstituted (Australia)							2.14	21.4	Knight et al., 1998 *(89)*
ISOMIL, reconstituted (Australia)							2.19	21.9	
KARICARE SOY, reconstituted (Australia)							1.72	17.2	
PROSOBEE, reconstituted (Australia)							1.75	17.5	
SOY FORMULA, ready-to-feed (New Zealand)	–	**18** (55%)	–	**15** (45%)	–	–	–	33	Irvine et al., 1998 *(90)*[h]
"FORMULA A" powdered, not reconstituted (New Zealand)	9.2	**[12.4]**[c] (62.6%)	5.5	**[7.4]**[c] (37.4%)	–	–	14.7	**[19.8]**[c]	Irvine et al., 1998 *(90; 91)*[h]
"FORMULA B" powdered, not reconstituted (New Zealand)	8.1	**[10.9]**[c] (61.8%)	5	**[6.7]**[c] (38.2%)	–	–	13.1	**[17.7]**[c]	

Table 9. (continued)

Formula Description	Isoflavones in Aglycone Equivalents								References[a]
	Genistein		Daidzein		Glycitein		Total Isoflavone		
	mg/100 g	Ready To Feed mg/L (% total)	mg/100 g	Ready To Feed mg/L (% total)	mg/100 g	Ready To Feed mg/L (% total)	mg/100 g	Ready To Feed mg/L	
"FORMULA C" powdered, not reconstituted (New Zealand)	9.1	**[12.3]**[c] (65.5%)	4.8	**[6.5]**[c] (34.5%)	–	–	13.9	**[18.8]**[c]	Irvine et al., 1998 *(90; 91)*[h]
"FORMULA D" powdered, not reconstituted (New Zealand)	8.3	**[11.2]**[c] (65.4%)	4.4	**[5.9]**[c] (34.6%)	–	–	12.7	**[17.1]**[c]	

Brand names are registered to their owners.

[a] While not presented here, studies included in the USDA database are assigned a Confidence Code (CC) of a, b, or c. The Confidence code is an indicator of relative quality of the data and the reliability of a given mean value. A confidence Code of "a" indicates considerable reliability, due either to a few exemplary studies or to a large number of studies of varying quality.

[b] CERHR calculated contribution of each isoflavone to the total based on other information in the paper, i.e., content of individual isoflavones or percentage distributions of individual isoflavones to the total.

[c] CERHR calculated as prepared (mg/L) from powdered formula based on the assumption that 0.135 kg of powder is used to reconstitute 1L of formula *(92)*. Sample calculation: 20.3 mg total isoflavone/100g powdered formula = 203.0 mg total isoflavone/kg powdered formula. Each liter of prepared formula then contains 27.4 mg total isoflavone.

[d] For formula published "as prepared" or "ready to drink," CERHR converted between mg/kg (or µg/g) and mg/L because the density of prepared formula is similar to water (specific gravity is 1.03) (information on specific gravity provided by personal communication from Mead Johnson Medical Nutrition Affairs).

[e] CERHR calculated by dividing the sum of individual isoflavones (aglycons+conjugates) presented in the published paper by Setchell et al.[6]the total isoflavone published value.

[f] **[For three of the five formulas presented in Setchell et al., 1998 [6], the published values were similar but not identical to the values calculated by CERHR from summing the published content of individual isoflavone aglycones and conjugates: MEAD JOHNSON, PROSOBEE, liquid concentrate, reconstituted (91 µg/g published value; 100.2 µg/g CERHR value); ROSS, ISOMIL, powder, reconstituted as fed (316.9 µg/g published value; 317.1 µg/g CERHR value); WYETH-AYERST, NURSOY, powder, reconstituted (307.3 µg/g published value; 311.2 µg/g CERHR value). Published values for the other 2 formulas presented in the paper were identical to those calculated by CERHR].**

[g] CERHR calculated as prepared (mg/L) from liquid concentrate based on the assumption that the reconstituted formula is a 2:1 dilution. Sample calculation: 6 mg total isoflavone/100g powdered formula = 60 mg total isoflavones/L liquid concentrate. Each liter of prepared formula then contains 30 mg total isoflavone.

[h] The Irvine et al., 1998 publications[90; 91]present summary tables for 4 brands of formula, however, the methods section for *(91)* states that 5 soy infant soy formula brands were tested ("Four commonly used brands of powdered, soy-based infant formula and one liquid, ready-to-feed brand were used."). Based on the units in the summary table (mg/kg), the 4 described brands were assumed by CERHR to be powdered, not reconstituted. The description of the "ready-to-feed" soy formula appears to be presented in the text of *(90)* as "By contrast, the ready-to-feed soy formula contained total genistein and daidzein concentrations of 18 and 15 µg/ml, respectively."

The UK Ministry of Agriculture, Fisheries, and Food summarized and compared levels of isoflavones and their conjugates measured in soy formula from other countries, and concluded that isoflavone levels in soy formula from the UK were similar to concentrations reported in other countries; differences most likely resulted from batch variations in soy isolate isoflavone levels, slight variations in formulas, and minor differences in analytic methodology. A comparison of levels of isoflavones in formula normalized to "as prepared" from different countries is presented in **Table 10**. The lowest total isoflavone content of 10 mg/L was measured in a sample of Nestle Alsoy powdered formula purchased in São Paulo by Genovese *et al.*, 2002 *(88)* and the highest, 47 mg/L, was reported for a sample of Abbott Nutrition (formerly Ross), by Setchell *et al.*, 1998 *(6)*.[7]

The isoflavone content of soy-based infant formulas are hundreds of times greater than those reported for casein-based formula (cow or goat milk) or breast milk (**Table 11**). Based on formula samples obtained in the UK, Kuhnle *et al.*, 2008 *(65)* reported the total isoflavone content of the soy infant formula as ~1,000 times higher than in the non-soy infant formula **[brand names are not provided; the formula is described as "baby formula powder"]**. The isoflavone content of cow's milk is higher than a casein-based formula, but still much lower than a soy-based infant formula (**Table 11**).

[7] A higher isoflavone content of 93 mg/L, based on the sum of genistein and daidzein, was presented for liquid Korean soy infant formula by Lee *et al.*, 2003 [93] **[4.67 mg/100 ml genistein and 4.64 mg/100 ml of daidzein, equal to 46.7 mg/L genistein and 46.4 mg/L daidzein for a combined isoflavone content of 93 mg/L]**. The study also measured the isoflavone content in samples of casein-based formula and breastmilk. In addition, the authors reported urine and plasma concentrations in infants fed soy formula (n=10), casein-based formula (n=14), or breast milk (n=15) for 4 months. Infants in the 3 groups were also compared on weight, length, head circumference, chest circumference, and infant development based on the developmental quotient. However, the main text of the article is written in Korean and many specifics of the study are unknown. Moreover, the isoflavone levels presented in the publication raise concerns on the reporting quality of the study. The value of 93 mg/L in soy formula is approximately twice the amount of the next highest reported level, 47 mg/L by Setchell *et al.*, 1998 [6]; a difference that also represents the typical dilution factor for liquid concentrates which raises uncertainty on whether the liquid formula sample was a "ready-to-feed" sample or a liquid concentrate. **[A visiting scientist at the NIEHS translated the article for CERHR but it did contain additional information on the brand name, type of sample, or number of samples tested]**. Lee *et al.*, 2003 also presented information on serum and urine isoflavone levels in soy formula fed infants, but the presented values are difficult to reconcile with other estimates as the blood-based estimates are lower (plasma=392.1 ng/ml verus 684 from Setchell *et al.*, [84] and 757 from Cao *et al.*, 2009 [94], but the urine values much higher (urine=17.89 µg/ml [17,900 ng/ml] verus 5891 ng/ml from Cao *et al.*, 2009 [94]. Additional concerns are raised about the study because the reported concentrations of genistein + daidzein in breast milk were much higher, 0.16 mg/100ml **[1.6 mg/L, equal to 1600 µg/L or ng/ml]** than any other reported value including a total isoflavone level of 32 ng/ml from breast milk samples collected from vegan women as described in Mortensen *et al.*, 2009 [14]. Yet, despite the much higher reported breast milk levels, the plasma levels of genistein and daidzein in the breastfed infants in the Lee *et al.*, 2003 study, 3.8 and 3.4 ng/ml, were similar to the whole blood levels reported in Cao *et al.*, 2009 [94] (10.8 ng/ml of genistein, 5.3 ng/ml of daidzein) and plasma by Setchell *et al.*, 1997 [84] (2.8 ng/ml of genistein and 1.5 ng/ml of daidzein). CERHR attempted to contact the corresponding author but was not able to obtain clarification on the study. Because of the uncertainty in the published values, the Lee *et al.*, 2003 [93] is not considered in the Expert Panel's evaluation of soy formula.

Table 10. Comparison of Isoflavone Content in Infant Soy Formulas from Different Countries

Country	*Total Isoflavones, mg/L formula as fed*[1]	*Individual Isoflavones, mg/L formula as fed,*[1] *% of total*			*Reference*
		Genistein	*Daidzein*	*Glycitein*	
US (n=20)	20.9[a] –47[b]	12.1[a] –~31.2[b] (57.7%–~66.3%)	7.1 a –~13.5[b] (~28.8%–34%)	1.7[a] –~2.4[b] (~5%–8.3%)	[a] Franke et al., 1998 *(81)* [b] Setchell et al., 1998 *(6)*
UK (n=13)	18[a] –46.7[b]	10.4[a] –31.3[b] (58.0%–67.1%)	6.7 a –12.5[b] (26.8%–37%)	0.9[a] –2.8[b] (5%–6.1%)	[a] UK Ministry of Agriculture, Fisheries, and Food *(5)* [b] Hoey et al., 2004 *(87)*
Australia (n=4)	17.2–21.9	NS	NS	NS	Knight et al., 1998 *(89)*
New Zealand (n=5)	17.1–33	11.2–18 (55%–65.4%)	5.9–15 (34.6%–45%)	NR	Irvine et al., 1998 *(91)*
Brazil (n=7)	10–27.4	5.9–16.2 (59.3%–59.8%)	2.4–8.6 (23.9%–31.7%)	1.6–2.4 (9%–16.3%)	Genovese et al., 2002 *(88)*

[1] CERHR converted between mg/kg (or µg/g) and mg/L because the density of prepared formula is similar to water (specific gravity is 1.03) Personal communication from Mead Johnson Medical Nutrition Affairs.
NS=Not specified; NR = Not reported

Table 11. Isoflavone Concentrations in Samples of Soy-Based Formula, Cow's Milk-Based Formula, Cow's Milk, or Human Breast Milk

Country		*Total Isoflavone, mg/L*[a]	*Reference*
Soy-Based Formula			
US, UK, NZ, Australia, Brazil (**Table 9**)		10–47	Genovese et al., 2002 *(88)*, Setchell et al., 1998 *(6)*
Casein-Based Formula			
NZ		< LOD	Irvine et al., 1998 *(90; 91)*
US		< LOD	Setchell et al., 1998 *(6)*
Australia		0.001–0.03	Knight et al., 1998 *(89)*
UK		0.08[b]	Kuhnle et al., 2008 *(65)*
Breast Milk			
Chinese (n=1)	Baseline Post-soy challenge	0.035 (35) 0.052 (52)	Franke et al., 1998 *(81)* as cited in Mortensen et al., 2009 *(14)*
US, omnivorous (n=9)		0.0056, mean	Setchell et al., 1998 *(6)*
US (n=1)	Baseline Post-soy challenge	0.001 ~0.0265	Setchell et al., 1998 *(6)*
US (n=7)	Baseline Post-soy challenge	0.0013, mean 0.0185, mean	Franke et al., 2006 *(95)* as cited in Mortensen et al., 2009 *(14)*
UK, omnivorous (n=14)		0–0.002, range	Friar and Walker *(96)* as cited in Mortensen et al., 2009 *(14)*; *(3)*
NZ (n=11, 2 were vegetarian)		< LOD (50 ng/L)	Irvine et al., 1998 *(91)*
UK, vegetarian (n=14)		0.001 – 0.010, range	Friar and Walker *(96)* as cited in Mortensen et al., 2009 *(14)*; *(3)*
UK, vegan (n=11)		0.002–0.032, range	

Table 11 (continued)

Country	Total Isoflavone, mg/L	Reference
Cow's Milk		
Australia	0.05–0.350	King et al., 1997 as cited in Mortensen et al., 2009 *(14)*
France	0.005–0.032	Antignac et al., 2003 as cited in Mortensen et al., 2009 *(14)*

Modified from Mortensen et al., 2009 *(14)*.
[a] Total is based on genistein and daidzein only.
[b] CERHR calculated as prepared (mg/L) from powdered formula based on the assumption that 0.135 kg of powder is used to reconstitute 1L of formula *(92)*.

Mortensen *et al.*, 2009 *(14)* recently reviewed the literature on isoflavone content in breast milk, cow's milk-based infant formula, and cow's milk. Two of the reviewed studies measured total isoflavone in cow's milk and reported ranges of 0.05-0.350 mg/L (King *et al.*, 1997 as cited in Mortensen *et al.*, 2009 *(14)*) and 0.005-0.032 mg/L (Antignac *et al.*, 2003 as cited in Mortensen *et al.*, 2009 *(14)*). The isoflavone content of breast milk is discussed further in **Section 1.2.2.6**.

Weaning foods can also be another source of isoflavone exposure in infants. The UK Committee on Toxicity *(3)* reported total isoflavone levels in "weaning foods," which included 22–66 mg/kg in instant weaning foods and 18–78 mg/kg in ready-to-eat weaning foods (**Table 12**). **[Genistein levels were not quantified separately. Examples of weaning foods examined were not provided, and it is not known if similar weaning foods are available in the US.]** Irvine *et al.*, 1998 *(90)* measured genistein and daidzein in 3 different infant cereals [composition not indicated] and 2 different infant dinners [composition not indicated] purchased in New Zealand, genistein + glycoside levels were measured at 3–287 mg/aglycone equivalents/kg food and daidzein + glycoside levels at 2–276 mg/aglycone equivalents/kg food *(90)*. **[It is not known if similar cereals and dinners are available in the US.]**

Table 12. Concentration of Isoflavones in Soy-Containing Weaning Foods

Food	Total Genistein, mg/kg	Total Daidzein, mg/kg	Total Isoflavone, mg/kg	Reference
Infant cereals (n = 3)	3–287	2–276	**[5–563]**[c]	Irvine et al., 1998 *(90)*[a]
Infant dinner (n = 2)	32–58	31–45	**[63–103]**[c]	
Rusks	< 0.1	< 0.1		
Instant weaning foods			22–66	UK Committee on Toxicity, 2003 *(3)*[b]
Ready to eat weaning foods			18–78	
Soy yogurts			29–83	
Soy milk			130–300	
Soy dessert			104	
Firm tofu			275	

[a] Data on infant cereals, infant dinners, and rusks were obtained from food products used in New Zealand *(90)*.
[b] Modified Table 4.8 from the UK Committee on Toxicity report "Phytoestrogens and Health"*(3)*.
[c] CERHR calculated based on sum of genistein and daidzein.

The study authors noted that a single serving of 1 of the cereals could result in isoflavone exposures ranging from 0.01 to 0.8 mg aglycone equivalents/kg bw/day in a 4-month-old infant, thus potentially increasing isoflavone intake by more than 25% compared to the same aged infant on a soy-based infant formula (estimated to be 2.9 mg aglycone equivalents/kg bw in this study).

Brief descriptions of studies reporting isoflavone levels in infant soy formula

In studies supported by Wyeth Laboratories, Protein Technologies International, and the National Institutes of Health (NIH), Setchell *et al.*, 1997 *(84)* measured isoflavone levels in infant soy formulalaand in the blood of infants (n=7) consuming soy formula. Results of the study, as well as additional details about methodology, were published in a later report by Setchell *et al.*, 1998 *(6)*. Following extraction with methanol, isoflavone levels in 5 US infant formulas were measured by high performance liquid chromatography (HPLC). Genistein and daidzein conjugates, mainly glycosides, were the most abundant isoflavone-related compounds identified. Mean percentages of isoflavones and their respective conjugates were reported at 67.1% genistein equivalents, 28.7% daidzein equivalents, and ~5% glycitein equivalents in soy infant formula. Levels of individual isoflavones, based on conversion to aglycone concentrations, are presented in **Table 9**. The total isoflavone content ranged from 2.6–4.1 mg/100 g formula as prepared. When converted to mg/L, these values, ~26–41mg/L, are similar to those reported by Setchell *et al.*, 2007 *(84)*. Estimated infant exposures resulting from soy formula intake are summarized in **Table 26**.

Franke *et al.*, 1998 *(81)* measured isoflavone + conjugate levels in 4 US brands of soy formula using methanol extraction and HPLC with diode-array ultraviolet detection. Isoflavone conjugation patterns were similar to those found in soy foods and included malonates (32–43%), glucosides (37–52%), acetates (6–7%), and aglycones (9–13%). Total isoflavones were measured at 155.1–281.4 µg/g. Percentages of each type of isoflavone included 55.3–57.7% genistein equivalents, 34–36.1% daidzein equivalents, and 7.4–8.5% glycitein equivalents. Based on an intake of 1 L formula, a body weight of 4.5 kg, and instructions for preparing formula, the authors estimated infant isoflavone equivalents exposure at ~7 mg/kg bw/day (). The authors stated that isoflavone + conjugate exposure in infants fed soy formula is 4–6 times higher than in adults eating a soy-rich diet (~30 g/day).

Irvine *et al.*, 1998 *(90)* used an HPLC technique to measure isoflavone equivalent levels in infant soy formulas and foods such as cereals and pureed meats or vegetables purchased in New Zealand. In both soy formula and infant foods, the majority of genistein and daidzein were present as their respective glucosides. In soy formula, total concentrations of isoflavones (mean ± SEM) were 87 ± 3 µg/g genistein and 49 ± 2 µg/g daidzein. [**If it is assumed that genistein and daidzein are the only isoflavones in the formulas, the percentages of total isoflavone represented by each compound are 64% genistein equivalents and 36% daidzein equivalents.]**

Murphy *et al.*, 1997 *(82)* analyzed 6 brands of soy formula sold in the US and reported that total isoflavone levels were similar across brands and were comprised of 59% genistein equivalents, 29% daidzein equivalents, and 12% glycitein equivalents. Levels of isoflavones and their conjugates are reported in the USDA survey and summarized in **Table 9**.

Hoey *et al.*, 2004 *(87)* used a liquid chromatography-mass spectrometry (LC-MS) method to measure isoflavone + conjugate levels in 3 soy-based infant formulas from the UK. The total isoflavone content of soy formulas consisted of ~58–67% genistein equivalents, 27–29% daidzein equivalents, and

6–16% glycitein equivalents. The study authors estimated that 4–6-month-old infants consumed between 17.5 and 33.0 mg/day isoflavone equivalents or 1.7–4.4 mg aglycone equivalents/kg bw/day. Hoey *et al.*, 2004 *(87)* also measured isoflavone levels in cow milk-based infant formulas from the UK. Isoflavones were not detected in the majority of the cow-milk infant formulas (0.5 mg/kg detection limit). Infants fed the cow-milk formula with the highest isoflavone content (2.1 mg/kg formula) were estimated to consume 0.16–0.27 mg/day isoflavone equivalents, or 0.02–0.03 mg aglycone equivalents/kg bw/day.

The UK Ministry of Agriculture, Fisheries, and Food *(5; 96)* conducted a survey of isoflavone levels in 6 brands of infant soy formulas. The formulas were analyzed for 14 different isoflavones and their conjugates and 1 coumestan compound using methanol extraction followed by HPLC and LC-MS. Isoflavone levels were normalized to aglycone concentrations. Isoflavones were detected in all soy formulas at concentrations of 18–41 mg/L made-up formula. The majority of isoflavones were present as glycosides, but smaller amounts of acetyl and malonyl forms and aglycones were also present. The most abundant isoflavones, genistein-, daidzein-, and glycitein-related compounds, represented an average of 58, 36, and 6% of formulations on a molar basis, respectively. The isoflavones/conjugates glycitein, 6′-O-acetylglycitin, formononetin, and biochanin A and the coumestan coumestrol were not detected. Eight batches of 1 soy formula, SMA Wysoy, purchased at different times and locations were analyzed to determine variation between batches. Isoflavone concentrations were 18–33 mg aglycone equivalents/L formula as made up. The UK Ministry of Agriculture, Fisheries, and Food concluded that isoflavone levels did not vary enough to significantly impact exposure. Statistical analyses by analysis of variance (ANOVA) and *F*-test did not find a significant difference at the 5% level in isoflavone levels between batches of the same brand and between different brands. The UK Ministry of Agriculture, Fisheries, and Food *(96)* analyzed 3 cow-milk formulas for isoflavones using HPLC and LC-MS methods, and reported that isoflavone levels were below the detection limit of 0.25–0.5 mg/L

Genovese *et al.*, 2002 *(88)* used HPLC and photodiode array detection to measure isoflavone content of 7 brands of soy-based infant formula purchased in Brazil. Most isoflavones were present as either β-glycosides (53–71.7%) or malonylglycosides (11.6–42.7%) in all formulas; the percentage range of isoflavones detected as aglycones was 2.4 to 29.7%. In all brands, the relative ranking of isoflavones as proportion detected was total genistein > total daidzein > total glycitein.

1.2.2.6 Biological Monitoring of Isoflavones in Blood, Urine, Amniotic Fluid, and Breastmilk

Genistein and daidzein exist mainly as glycosides in unfermented soy foods. Due to their high water solubility and molecular weight, isoflavone glycosides are not readily absorbed across the gastrointestinal tract (reviewed in *(3)*). For absorption to occur, isoflavones must first be hydrolyzed to their aglycones, which have some hydrophobicity and lower molecular weights. Absorption occurs primarily in the small and large intestine. Isoflavones are readily absorbed as indicated by frequent detection in the blood or urine, including in populations that do not consume diets traditionally associated with high intake of soy foods.

Fetal and maternal samples during pregnancy

Seven studies, briefly described below, were identified that measured isoflavone levels in fetal (amniotic fluid or cord blood) and/or maternal blood samples during delivery (**Table 13**) [97-103].

Table 13. Isoflavones in Amniotic Fluid, Cord Blood, and Maternal Blood at Delivery

Population	n	Sample	Mean Concentration (range), nM [µg/L]				Reference
			Genistein	Daidzein	Equol	Total	
US-American Women							
Advanced maternal age < 20 weeks gestation	21	Amniotic Fluid	1.38 (median) (0.20–7.88) **[0.37 (0.19–7.89)]**	9.52 (median) (3.84–17.4) **[2.42 (0.98–17.39)]**			Engel et al., 2006 *(98)*
15–23 weeks gestation	53	Amniotic Fluid	1.69 (max = 6.54) **[0.4 (max = 1.77)]**	1.44 (max = 5.52) **[0.37 (max = 1.40)]**			Foster et al., 2002a *(99)*
15–23 weeks gestation	*Genistein:* n=51 *Daidzein*: n=39	Amniotic Fluid	1.08 (0.50–4.86) **[0.29 (0.51–1.31)]**	0.94 (0.50–5.52) **[0.24 (0.13–1.40)]**			Foster et al., 2002b *(100)*
Japanese Women							
At delivery, full-term	7	Maternal Plasma	83.9 (9.16–303) **[22.67 (2.48–81.88)]**	45.5 (2.04–243) **[11.57 (0.52–61.78)]**	71.1 (0.63–401) **[17.22 (0.15–97.1)]**	201[a]	Adlercreutz et al., 1999 *(97)*
		Cord Plasma	165 (39.8–417) **[44.59 (10.76–112.69)]**	58.8 (10.2–137) **[14.95 (2.59–34.8)]**	50.9 (0–267) **[50.9 (0–64.6)]**	275[a]	
		Amniotic Fluid	64 (11.4–212) **[17 (3.1–57)]**	67.8 (15.6–156) **[17.24 (3.97–39.6)]]**	68.0 (0.13–397) **[16.47 (0.03–96.1)]**	199[a]	
Delivery by c-section, full term	51	Cord Serum	71.8 (max = 325.3) **[19.40 (max = 87.91)]**	16.9 (max = 110.5) **[4.30 (max = 28.09)]**	3.7 (max = 23.9) **[0.89 (max = 5.8)**		Todaka et al., 2005 *(103)*
Delivery by c-section, full term	51	Maternal Serum	26.6 (max = 219.1) **[7.2 (max = 59.2)]**	7.1 (max = 63.7) **[1.8 (max = 16.2)]**	8.3 (max = 78) **[2.0 (max = 18.9)**		
At delivery, full-term	194	Cord Blood	126.9 (1.9–1297) **[34.3 (0.5–350.50)]**	38.6 (2.0–303) **[9.81 (0.51–77.04)]**	4.2 (2.1–169) **[1.01 (0.51–40.94)]**		Nagata et al., 2006 *(102)*
		Maternal Serum	116.5 (1.9–2223) **[31.48 (0.51–600.7)]**	50.2 (2.0–1182) **[12.76 (0.51–300.52)]**	6.5 (2.1–797) **[1.57 (0.51–193.1)]**		
Malasyian Women							
At delivery	300	Cord Plasma	15.9 **[4.3]**	3.5 **[0.89]**			Mustafa et al., 2007 *(101)*
		Maternal Plasma	6.1 **[1.65]**	4.1 **[1.04]**			

[a]Total isoflavonoid concentration presented by Adlercreutz et al., 1999 *(97)* was based on genistein, daidzein, equol, and *o*-desmethylangolensin. The values presented in this table are based on the sum of genistein, daidzein, and equol.

OOnly amniotic fluid measurements were identified for samples collected in the U.S. For genistein, the range of central tendency values (mean or median concentration) was 1.08 nM **[0.29 µg/L]** to 1.69 nM **[0.46 µg/L]**. For daidzein, the range was 0.94 nM **[0.24 µg/L]** to 9.52 nM **[2.42 µg/L]**. These concentrations were much lower than those reported in Japanese samples. In a study of Japanese women, Adlercreutz *et al.*, 1999 *(97)* reported mean amniotic fluid concentrations of genistein (64 nM **[17.3 µg/L])**, daidzein (67.8 nM **[17.2 µg/L]**), equol (68.0 nM **[16.5 µg/L]**) and *O*-desmethylangolensin (23.6 nM **[6.1 µg/L]**) in amniotic fluid samples collected at delivery. The Expert Panel did not identify any other reports of equol or *O*-desmethylangolensin in amniotic fluid samples.

Foster *et al.*, 2002 *(100)* measured genistein and daidzein in 57 human amniotic fluid samples collected between 15 and 23 weeks of gestation. Samples were collected in Los Angeles **[ethnic composition and dietary factors not discussed]**. Measurements were made by GC/MS after glucuronidase treatment to hydrolyze the conjugates. Genistein was measurable in 42 of the samples (89.5%) with a mean ± SD concentration of 1.08 ± 0.91 ng/mL **[4.0 ± 3.4 nM]** (range 0.5–4.86 ng/mL **[1.5–17.9 nM]**). Daidzein was detected in 32 of 57 samples (68.4%) with a mean ± SD concentration of 0.94 ± 0.91 ng/mL **[3.7 ± 3.6 nM]** (range 0.5–5.52 ng/mL **[2.0 - 21.7 nM]**

In a different paper *(99)*, these authors reported genistein concentrations in 59 amniotic fluid samples obtained from 53 pregnant women at 15–23 weeks of gestation (4 sets of twins and 1 woman who was sampled 3 times). There were 42 women with measurable amniotic fluid genistein concentrations. The mean ± SD genistein concentration was 1.69 ± 1.48 ng/mL **[6.25 ± 5.48 nM]** (maximum 6.54 ng/mL **[24.2 nM]**). For daidzein, the mean ± SD genistein concentration was 1.44 ± 1.34 ng/mL **[5.66 ± 5.27 nM]** (maximum 5.52 ng/mL **[21.71 nM]**). **[In Table 2 of the publication, the mean ± SD genistein is reported as 1.37 ± 1.00 ng/mL (5.07 ± 3.7 nM) with a median of 0.99 ng/mL (3.7 nM). The mean ± SD daidzein is reported as 1.14 ± 1.00 ng/mL (4.48 ± 3.93 nM) with a median of 0.75 ng/mL (2.95 nM). It is not known whether there are any samples represented in both papers.]**

Adlercreutz *et al.*, 1999 *(97)* used a GC/MS method to measure maternal plasma, cord plasma, and amniotic fluid phytoestrogen levels in 7 healthy omnivorous Japanese women (20–30 years old) who had just given birth. Total genistein levels in maternal blood and unconjugated and conjugated levels in cord plasma and amniotic fluid are summarized in **Table 14.**Genistein, daidzein, equol, and *O*-desmethylangolensin were detected in cord blood and amniotic fluid, and levels were reported to be variable between subjects. Correlations between maternal blood and cord blood or amniotic fluid genistein levels were not statistically significant. Most of the genistein, daidzein, or equol measured in amniotic fluid or cord blood was represented by glucuronide or sulfoglucuronide conjugates (**Table 14**). **[Unconjugated and sulfate conjugates of genistein represented 10–15% of total genistein in cord blood and amniotic fluid.]** The study authors concluded that phytoestrogens cross the placenta.

Engel *et al.*, 2006 *(98)* measured genistein and daidzein in 21 amniotic fluid samples obtained prior to 20 weeks gestation. The samples were collected from women referred to the Mount Sinai Medical Center for the sole indication of "advanced maternal age" (>35 years). Measurements were made by use of an electrochemical detector in combination with HPLC. The median concentration (range) for genistein was 1.38 (0.20–7.88) µg/L and for daidzein was 9.52 (3.84–17.4) µg/L.

Table 14. Unconjugated, Conjugated, and Total Isoflavones in Amniotic Fluid and Cord Blood

Isoflavone	*Sample*	*Mean concentration (range), nM [µg/L]*			
		Unconjugated	*Conjugated (Glucuronides+ Sulfoglucuronides)*	*Total*	*Percent of Total as Unconjugated*
Genistein	Maternal plasma			83.9 (9.16–303)	
	Cord plasma	15.7 (3.51–37.3) **[4.2 (0.95–10)]**	150 (35.6–387) [41 (9.6–105)]	165 (39.8–417) [45 (11–113)]	9.5%
	Amniotic fluid	10.2 (2.93–24.4) **[2.8 (0.79–6.6)]**	53.8 (3.86–198) [15 (1.0–54)]	64 (11.4–212) [17 (3.1–57)]	15.9%
Daidzein	Maternal plasma			45.5 (2.04–243)	
	Cord plasma	7.94 (1.09–15.1) **[2.02 (0.28–3.84)]**	50.8 (8.84–122) **[12.92 (2.25–31.02)]**	58.8 (10.2–137) **[14.95 (2.59–34.8)]**	13.5%
	Amniotic fluid	13.5 (2.11–29.4) **[3.43 (0.54–7.47)]**	54.3 (5.03–143) **[13.81 1.28–36.36]**	67.8 (15.6–156) **[17.24 (3.97–39.66)]**	19.9%
O-Desmethylangolensin	Maternal plasma			31.2 (1.25–194)	
	Cord plasma	11.2 (1.02–48.9)	12.9 (0–60.8)	24.1 (1.02–110)	46.5%
	Amniotic fluid	3.09 (0–6.0)	20.5 (0.25–92.5)	23.6 (2.86–98.5)	13.1%
Equol	Maternal plasma			71.1 (0.63–401)	
	Cord plasma	11.5 (0–48.0) **[2.76 (0–11.6)]**	39.4 (0–219) **[9.5 (0–53.8)]**	50.9 (0–267) **[12.3 (0–64.7)]**	22.6%
	Amniotic fluid	3.80 (0–12.1) **[0.93 (0–2.93)]**	64.2 (0.13–385) **[15.5 (0.03–93.3)]**	68.0 (0.13–397) **[16.5 (0.03–96.18)]**	5.6%
Total isoflavones	Maternal plasma			232 (19.2–744)	
	Cord plasma	46.4 (10.1–96.6)	253 (47.4–735)	299 (57.8–831)	15.5%
	Amniotic fluid	30.6 (6.77–65.2)	193 (26.8–740)	223 (51.5–779)	13.7%

To convert from nM to µg/L unconjugated equivalents, multiple by: genistein 0.27, daidzein 0.25, *O*-desmethylangolensin 0.26, and equol 0.25.
From Adlercreutz et al., 1999 *(97)*.

Nagata *et al.*, 2006 *(102)* evaluated isoflavone exposure in 194 Japanese women by estimating the dietary intake and measuring of genistein, daidzein and equol in maternal urine, serum during gestation and at delivery, as well as serum in umbilical cord samples. Isoflavones were measured using HPLC-MS/MS (for serum) or HPLC (urine) after the addition of β-glucuronidase/sufatase to the samples. The geometric mean and range of concentrations of genistein, daidzein, and equol for maternal and cord blood serum samples are presented in **Table 13**. Urine concentrations measured during pregnancy and at delivery are reported in **Section 1.2.2.6**. Genistein and daidzein in umbilical cord serum were highly correlated with those in both maternal urine (r=0.63, $P<0.0001$ and r=0.58, $P<0.0001$ for genistein and daidzein, respectively, after controlling for covariates) and serum (r=0.70, $P<0.0001$ and r=0.68, $P<0.0001$ for genistein and daidzein, respectively after controlling for covariates) at delivery. **[Equol correlations were not reliable due to low detection frequency (less than 50% of samples were above LOD).]** The authors also reported correlations between isoflavones in maternal samples and cord blood with hormone levels of estradiol, estriol, and testosterone (discussed in **Section 3.4**). In umbilical cord blood, no significant correlation between the hormone and isoflavones were reported. There were a few significant associations between maternal hormone levels and isoflavones during pregnancy, but the pattern varied depending on stage of pregnancy and whether isoflavone exposure was based on dietary records or measured concentrations in urine or serum.

Mustafa *et al.*, 2007 *(101)* measured free and conjugated genistein, genistin, daidzein, daidzin, and coumesterol in 300 cord blood samples collected in Malaysia. Samples were collected from women who delivered babies in seven Malaysian hospitals located in urban and rural locations. In addition, 103 matching pairs of maternal and cord blood samples were analyzed to compare the distribution of isoflavones in maternal and cord plasma. Total isoflavone (conjugated and free forms) concentrations were determined by LC-MS preceded by solid-phase extraction using C18 column and passage through DEAE sephadex gel. The mean concentrations of total phytoestrogens in cord plasma samples were genistein (3.7 ± 2.8 ng/ml), genistin (19.5 ± 4.2 ng/ml), daidzein (1.4 ± 2.9 ng/ml), daidzin (3.5 ± 3.1 ng/ml), and coumesterol (3.3 ± 3.3 ng/ml) **[The authors state that concentrations of both free and conjugated isoflavones were measured. However, the reported values are presented as "total" concentration for each isoflavone and information on the relative distribution of free and conjugated, i.e., the percent of total as free, was not presented in the paper.]** Although no statistical analyses are presented, the distribution of phytoestrogen was reported as higher in samples collected from rural areas compared to that of urban areas. The concentrations of isoflavones measured in the matching maternal and cord plasma samples were very similar **[no correlations or other statistical analyses are reported].** The highest concentrations of phytoestrogens were detected in women who reported being daily consumers of bean sprouts (referred to as "taugeh" in Malaysia) and the lowest was in consumers of soy-based and soy-based milk. The authors did not conclude that taugeh was the main source of isoflavones compared to soy foods because of imbalances in sample size between food consumption groups.

Todaka *et al.*, 2005 *(103)*, funded by the Japanese Ministry of the Environment and the Ministry of Education, Culture, Sports, Science, and Technology, investigated the concentrations of phytoestrogens in maternal and cord blood serum in women giving birth by cesarean section. Their investigation involved 51 mother-infant pairs in which they determined concentrations of total genistein, daidzein, equol, and coumestrol. They also determined sulfate conjugated genistein concentrations in 10 of these mother-infant pairs. Because the mothers fasted for 15 hours prior to surgery, time for substantial

metabolism and excretion of these phytoestrogens, they also analyzed these phytoestrogens in the serum of 10 males and 10 females to determine levels in adults soon after breakfast. Analyses were done using electron spray ionization-liquid chromatography/tandem mass spectrometry (LC-MS/MS). The limit of detection was 0.5 ng/mL and data below this limit were considered zero. 4'-OH and 7-OH sulfate conjugated genistein was analyzed using a HPLC-UV apparatus.

Total genistein was detected in 100% of cord bloods with a mean concentration of 19.4 ng/mL, and in 96% of maternal bloods with a mean concentration of 7.2 ng/mL. Total daidzein was detected in 80% of cord bloods with a mean concentration of 4.3 ng/mL, and in 75% of maternal bloods with a mean concentration of 1.8 ng/mL. Total equol was detected in 35% of cord bloods with a mean concentration of 0.9 ng/mL, and in 37% of maternal bloods with a mean concentration of 2.0 ng/mL. Total coumestrol was not detected in any cord bloods and in only one maternal blood. Free (unconjugated) genistein, daidzein, equol, and coumestrol (detection limit=0.5 ng/mL) were not detected in any serum samples from maternal or cord blood.

There was no difference in the serum concentrations of phytoestrogens in male and female newborns and no correlation between serum concentrations and birth weights. Total genistein and daidzein concentrations were significantly higher in cord serum than in maternal serum while equol concentrations were significantly higher in maternal serum than in cord serum. Among the 10 mother-infant pairs analyzed for sulfate-conjugated genistein, this conjugated form was found in only one maternal serum sample but was detected in 8 cord samples (mean=5.2 ng/mL) and constituted about 10.7% of total genistein. Among the 10 male and 10 female adult volunteers sampled, mean phytoestrogen concentrations (ng/mL) were: total genistein=94.7 and free genistein=2.4, total daidzein=26.0 and free daidzein=2.1, total equol=4.0 and no free equol was detected. These levels are substantially higher than in maternal blood serum samples that were collected following 15 hours of fasting. The author's concluded that phytoestrogens are transferred from mother to fetus and that metabolic and/or excretion rates of phytoestrogens are different between mother and fetus and tend to stay longer in the fetal side than in the maternal side.

Infants

Several studies, briefly described below, have compared isoflavone levels in infants who are fed soy formula, cow's milk formula, or breast milk (summarized in **Table 15**). The highest blood-based concentrations of genistein were measured in a sample of 27 soy formula-fed infants from the US *(94)*. The geometric mean and median concentrations of genistein in whole blood were 757 and 890.7 ng/ml, respectively. At the 75th percentile, the genistein concentration was 1455.1 ng/ml. The mean concentration reported by Cao *et al.*, 2009 *(94)* is higher than the previous blood-based measurement of genistein in infants fed soy formula reported by Setchell *et al.*, 1997 *(84)* of 684 ng/ml in plasma samples from seven infants.

Table 15. Summary of Isoflavone Levels in Infants Based on Feeding Method

Population	Sample	n	Age, months	Isoflavone Concentrations (Total, Conjugated + Unconjugated), ng/ml[a, b, c]				Reference
				Genistein	Daidzein	Equol	Total	
Soy Formula-Fed								
US	Whole blood	27	0–12	757 ± 3.0[b] 890.7 (405.3–1455.1)[c]	256 ± 2.8[b] 274.8 (178.5–518.7)[c]			Cao et al., 2009 *(94)*
US	Plasma	7	4	684 ± 443[a]	295 ± 59.5[a]	~2.0 (ND –~3.5)		Setchell et al., 1997 *(84)*
US	Plasma	3	9–24				276 ± 106	Franke et al., 2006 *(95)*
US	Urine	125	0–12	5891 ± 3.1[b] 7220 (3390–11,300)[c]	5097 ± 2.5[b] 5590 (3630–8070)[c]	2.3 ± 4.6[b]		Cao et al., 2009 *(94)*
US	Urine	8	2	26,451 ± 8559[a]	25,399 ± 8559[a]			Venkataraman et al., 1992 as cited in Cao et al., 2009 *(94)*
		7	4	8,758 ± 3,808[a]	17,577 ± 5452[a]			
US	Saliva	119	0–12	11.6 ± 5.1[b] 10.9 (4.0–33.5)[c]	5.2 ± 5.8[b] 6.2 (1.0–18.3)[c]			Cao et al., 2009 *(94)*
NZ	Urine	4	0.5–4	0.15–0.32 mg/kd/day[d]	0.37–0.58 mg/kd/day[d]			Irvine et al., 1998 *(91)*
Cow Milk Formula-Fed								
US	Whole blood	30	0–12	14.2 ± 1.5[b]	5.5 ± 1.5[b]			Cao et al., 2009 *(94)*
US	Plasma	7	4	3.16 ± 0.68[a]	2.06 ± 0.29[a]	4.11 ± 0.49[a]		Setchell et al., 1997 *(84)*
US	Urine	128	0–12	11.8 ± 5.7[b] 13.6 (5.6–32.4)[c]	8.2 ± 5.0[b] 10.6 (2.6–22.4)[c]	2.4 ± 2.1[b]		Cao et al., 2009 *(94)*
US	Urine	5	2	205 ± 52[b]	155 ± 64[b]	42.6 ± 2.3[a]		Venkataraman et al., 1992 as cited in Cao et al., 2009 *(94)*
		6	4	536 ± 393[b]	706 ± 555[b]	40.5 ± 3.3[a]		
NZ	Urine	25	0.5–4	Under limit of detection				Irvine et al., 1998 *(91)*
US	Saliva	120	0–12	0.7 ± 1.3[b]	0.4 ± 1.4[b]			Cao et al., 2009 *(94)*

Table 15 (continued)

Population	Sample	n	Age. months	Isoflavone Concentrations (Total, Conjugated + Unconjugated), ng/ml[a,b,c]				Reference
				Genistein	Daidzein	Equol	Total	
Breastfed								
US	Whole blood	20	0–12	10.8 ± 2.7[b]	5.3 ± 1.2[b]			Cao et al., 2009 *(94)*
US	Plasma	7	4	2.77 ± 0.73[a]	1.49 ± 0.13[a]	~0.50 (ND–~3.5)[a]		Setchell et al., 1997 *(84)*
US	Plasma	11	0.5–1	**[3.19]**[e]	**[2.00]**[e]		**[5.19]**[b,c,e]	Franke et al., 2006 *(95)*
	Urine	7	0.5–11				29.8 ± 11.6 nmol/mg creatinine[a]	
US	Urine	128	0–12	1.5 ± 4.8[b]	1.5 ± 2.9[b]	1.7 ± 1.2[b]		Cao et al., 2009 *(94)*
US	Urine	5	2	1284 ± 1089[a]	697 ± 653[a]	2.9 ± 2.9[a]		Venkataraman et al., 1992 as cited in Cao et al., 2009 *(94)*
		5	4	161 ± 108[a]	179 ± 80[a]	59 ± 42[a]		
US	Saliva	120	0–12	0.7 ± 1.3[b]	0.4 ± 1.5[b]			Cao et al., 2009 *(94)*
Following Soy Challenge								
US After infant ingestion of 9–25 g tofu	Plasma	3	9–25	**[170.02]**[e]	**[106.6]**[e]		**[276.7]**[b,c,e]	Franke et al., 2006 *(95)*
	Urine	3	9–2 (n=3)				229 nmol/mg creatinine[a]	
US Breastfed infants after maternal daily consumption of soy protein beverage (36.5g) for 2–4 days	Urine	7	0.5–11				111.6 ± 18.9 nmol/mg creatinine[a]	

*1 nM = 0.270 µg/L or 270.24 ng/L genistein, 0.254 µg/L or, 0.284 µg/L or 284.26 ng/L equol, and 0.284 µg/L or 284.16 ng/L glycitein. Conversions in the table refer to aglycone equivalents.

ND = Not detected

[a] Mean ± SD

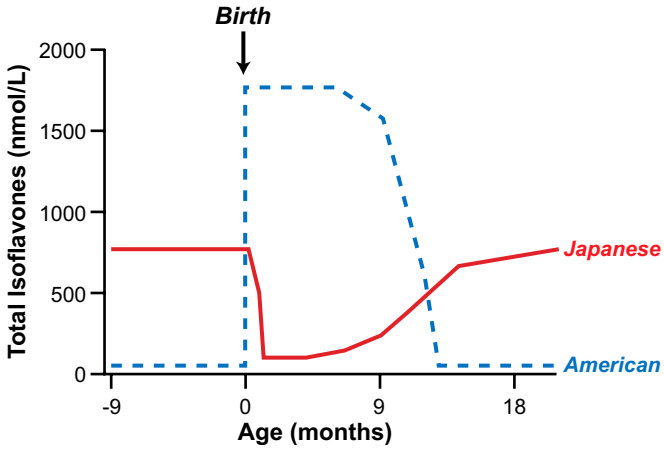
[b] Geometric mean ± Geometric SD

[c] Median (25th–75th percentile)

[d] Range of mean excretion rate in individual infants.

[e] Frank et al., 2006 *(95)* present total isoflavone in nmol/L and state that 40% of the total in infant plasma was daidzein. CERHR used this ratio to estimate the proportion of total as genistein and daidzein and these estimates of genistein and daidzein were used to estimate a total isoflavone concentration in µg/L.

Figure 2. Hypothetical Comparison of Serum Isoflavone Levels from Conception Through Weaning in Typical Asians and Soy-Formula-Fed Infants in the US



Modified from Figure 1 in Badger et al., 2002 *(33)*.

Differences in soy food exposure patterns throughout life were noted for Americans compared to Asians *(33)* (**Figure 2**). In the US, typical diets are low in soy food intake, and the fetus is thus exposed to low levels of genistein. Significant exposure to genistein and its conjugates occurs in the approximately 25% of infants who are fed soy formula. After those infants are weaned, soy food intake and genistein exposure drops and typically remain low over the lifetime. In Asian cultures consuming soy products, the fetus is exposed to genistein and its conjugates as a result of maternal soy food intake. At birth, most infants are either breast fed or fed cow-milk formula, so exposure to genistein is very low during infancy. Upon weaning, the infants begin receiving soy foods and exposure to soy products and genistein + conjugates remain high over their lifetime.

Cao *et al.*, 2009 *(94)* measured the isoflavones in the blood, saliva, and urine of 166 full-term infants who were fed soy formula, cow milk formula, or breast milk. Automated online solid-phase extraction combined with HPLC-MS/MS was used for urinary measurements and LC/MS/MS was used for saliva and blood spots. Daidzein and genistein were detected in the blood, saliva, and urine of the majority of infants on soy formula. Both the measured concentration and detection frequency of daidzein and genisten in these infants were higher for urine than blood or saliva (**Table 15** and **Table 16**). For example, all the samples from soy formula fed infants had detectable levels of daidzein or genistein in urine but the percent of samples with detectable levels in blood or saliva ranged from 83 to 96%. In contrast, the majority of infants fed cow's milk formula or human milk did not have detectable concentrations of daidzein or genistein in blood or saliva. Urinary concentrations of daidzein and genistein were approximately 500-times lower in these infants compared to infants on a soy formula diet. Equol was not detected in blood or saliva from any infant and was only detectable in a minority of urine samples (5% soy formula fed, 22% of cow milk formula fed, and 2% of breast milk fed infants). Other isoflavone metabolites, such as *O*-desmethylangolensin, were either not measured *(94)* or not detected *(84)*

Setchell *et al.*, 1997, 1998 *(6; 84)* used an enzymatic deconjugation process and a gas chromatography/mass spectrometry (GC-MS) method to measure plasma total isoflavone levels in seven 4-month-old male infants fed Isomil soy formula. Blood samples were obtained between 9 and 11 AM and

Table 16. Detection Frequency of Daidzein, Genistein, and Equol in Infants Fed Soy Formula, Cow Milk Formula, or Breast Milk

Isoflavone	*Sample*	*LOD (ng/ml)*	*Frequency Detection by Feeding Method, % detection ($n_{>LOD}$/ n_{total})*			*Reference*
			Soy Formula	*Cow Milk Formula*	*Breast Milk*	
Daidzein	Whole blood	10	96% (26/27)	3% (1/30)	5% (1/20)	Cao et al., 2009 *(94)*
	Saliva	0.76	83% (100/120)	7% (9/120)	7% (7/120)	
	Urine	1.6	100% (125/125)	78% (100/128)	30% (38/128)	
Genistein	Whole blood	27	96% (26/27)[a]	10% (3/30)	5% (1/20)	Cao et al., 2009 *(94)*
	Saliva	1.4	91% (109/120)	5% (6/120)	2% (3/121)	
	Urine	0.8	100% (125/125)	91% (116/128)	49% (63/128)	
Equol	Whole blood	12	0% (0/27)	0% (0/30)	0% (0/20)	Cao et al., 2009 *(94)*
	Saliva	3.6	0% (0/120)	0% (0/120)	0% (0/121)	
	Urine	3.3	5% (6/124)	22% (27/125)	2% (2/128)	
	Plasma		57% (4/7)	100% (7/7)	14% (1/7)	Setchell et al., 1997 *(84)*

[a] Table 4 in the Cao et al., 2009 publication states the number of blood samples with non-detectable levels of genistein in the soy formula-fed infant group as 0/27, or 4% < LOD. CERHR has confirmed that the number should be 1/27 < LOD for genistein in blood of soy group (personal communication with co-authors Yang Cao and David Umbach, October 13 and 15, 2009).

the infants were not fasted before collection. Mean ± SD plasma genistein was 683 ± 442.6 µg/L, and mean ± SD plasma daidzein was 295.3 ± 59.9 µg/L. Equol was detected in 4 of 7 infants in the soy formula group, 7 of 7 infants in the cow milk formula group, and 1 of 7 infants fed breast milk. Total isoflavones were reported at 552–1775 µg/L (mean 980 µg/L) **[Plasma glycitein levels were not measured.]** The study authors noted that they did not attempt to measure the extent of isoflavone conjugatio0n in infant serum. Total plasma isoflavone levels were 50–100-fold higher in infants fed soy formula compared to 4-month-old male infants fed breast milk (mean ± SD 4.7 ± 1.3 µg/L, n=7) and cow milk formula (mean ± SD 9.3 ± 1.2 µg/L, n=7). Plasma isoflavone levels in infants fed soy formula were also higher compared to adults (50–200 µg/L) and Japanese adults (40–240 µg/L) ingesting similar levels of isoflavones + conjugates from soy-based foods.

Franke *et al.* in two separate studies *(81; 104)* measured isoflavone and metabolite levels in the breast milk and urine of postpartum women who consumed roasted soybeans. Plasma levels were also examined in 1 study *(81)*. In the first study *(104)*, isoflavone levels were measured in soybeans and found to be (in aglycone equivalents): daidzein 830 mg/kg soybean, genistein 913 mg/kg soybean, and glycitein 174 mg/kg soybean. Authors estimated the 3 daidzein doses at 0.08, 0.15, and 0.30 mg/kg bw and the 3 genistein doses at 0.08, 0.17, and 0.33 mg/kg bw. In the second study *(81)*, the soybeans

reportedly contained daidzein 0.85 mg/kg soybean and genistein 1.1 mg/kg soybean, and the authors stated that consumption of 20 g soybeans would result in intake of 37 mg isoflavones. **[Based on the authors' value for isoflavone intake, it appears that the units of isoflavones in soybeans should have been mg/g soybean instead of mg/kg soybean, consistent with data reported for mature soybeans in the USDA *(11)* survey. Assuming that the correct unit for the isoflavones is mg/g soybean, 20 g soybeans would contain ~17 mg total daidzein and 22 mg total genistein.]** Milk samples were collected each time the infant was nursed, and blood samples were drawn in 1 study following an overnight fast. Isoflavones and metabolites were hydrolyzed enzymatically, extracted from samples using methanol or ethyl acetate, and analyzed by HPLC.

In both studies, isoflavone levels in milk were below the detection limit (1–3 pM) prior to hydrolysis, suggesting that isoflavones occur in milk as glucuronide and sulfate conjugates. Soybean intake resulted in a rapid and dose-dependent increase in genistein and daidzein derivatives in milk. Concentrations peaked 10–14 hours following consumption of soybeans and returned to baseline levels 2–4 days later. A biphasic pattern of isoflavone detection was observed in milk after consumption of 20 g soybeans. Milk samples did not contain glycitein or the metabolites equol and *O*-desmethylangolensin at detectable levels. Milk contained higher concentrations of genistein than daidzein conjugates. Levels of genistein conjugates were also higher in plasma **[data were not shown]**. In urine, the ratio of isoflavones to metabolites suggested preferential excretion of metabolites **[data were not shown]**. In contrast to results for milk and plasma, urinary conjugates of daidzein were higher than conjugates of genistein. Glycitein and the isoflavone metabolites equol and *O*-desmethylangolensin were also detected in urine.

In the Franke *et al.*,1996 *(104)* study, intake of 5, 10, and 20 g soybeans by a single subject resulted in maximum milk total genistein levels of 32, 46, and 71 nM and total daidzein levels of 16, 34, and 62 nM, respectively. The respective maximum urinary excretion rates for total genistein were 60, 300, and 300 nmol/hour and for total daidzein, 150, 500, and 600 nmol/hour. Milk and urine levels were compared to those of a Chinese woman who ate her usual diet, which included 1 serving/day of tofu soup. The woman's milk contained total genistein at 30–50 nM and total daidzein at 80–110 nM; urinary excretion rates ranged from 8 to 33 nmol/hour for total genistein and from 80 to 150 nmol/hour for total daidzein. In the Franke *et al.*, 1998 *(81)* study, mean total isoflavone concentrations after consumption of 20 g soybeans were 0.2 µM in milk and 2 µM in plasma; urinary excretion rate for isoflavone was 3.0 µmol/hour. **[The data for "total isoflavones" were presented in the study abstract.]**

Franke *et al.*, 2006 *(95)* compared isoflavones patterns in the urine and plasma of tofu-fed infants and breastfed infants and their mothers following maternal consumption of a soy protein beverage. Three infants consumed a tofu sample with an average of 7.4 mg isoflavones that resulted in a mean and standard deviation dose of 0.694 ± 0.42 mg/kg. Sixteen mothers drank a soy protein beverage once daily for 2 - 4 days that contained ~ 55 mg isoflavones (total daidzein:genistein:glycitein=1:1:0.1, mainly as glucosides and malonylglucosides) which resulted in an average body weight adjusted dose of 1.01 ± 0.03 mg/kg. Samples of mother's urine (n=16), breastmilk (n=16), infant urine (n=13), and infant plasma (n=11) were collected in the afternoon following maternal consumption of the soy beverage in the early morning. Daidzein, genistein, glycitein, equol, dihydrodaidzein, dihydrogenistein, and *O*-desmethylangolensin were measured in breast milk, maternal urine, infant urine, and infant plasma by HPLC with photodiode array detection followed by electrospray ionization ion trap mass spectrometry.

Levels of genistein, daidzein, and glycitein were not presented individually, although the authors reported that glycitein values were negligible in breastmilk, contributing ~2% towards the concentrations of total isoflavones. The daidzein-to-genistein ratio in breast milk was reported as 0.6 *(95)*. Isoflavone concentrations were significantly correlated within type of biological matrix for an individual and within mother-infant pair for breastfed infants: mother's milk versus mother's urine, r=0.661; mother's milk versus infant urine, r=0.775; mother's urine verus infant urine, r=0.863, and infant plasma versus infant urine, r=0.975.

Baseline urine and milk samples were available for seven mother-infant pairs. Based on these samples, statistically significant increases in mean (±SEM) isoflavone excretion rates were observed in mother's urine (18.4±13.0 verus 135±26.0 nmol/mg creatinine), mother's milk (5.1±2.2 versus 70.7±19.2 nmol/L), and infant urine (29.8±11.6 verus 111.6±18.9 nmol/mg creatinine) following the mothers ingestion of the soy protein beverage. Inclusion of study participants who only donated specimans following the intervention resulted in slightly higher but similar means: 157.1±18.5 nmol/mg creatinine for mother's urine, 95.4±19.6 nmol/L for mother's milk, and 186.1±25.1 nmol/mg creatinine. Plasma samples were available for 11 of the breastfed infants=19.7 nmol/L (median: 2.5nmol/L; range:0.2–148.5nmol/L). Compared to either the mothers fed a soy beverage or their breastfed infants the tofu-fed infants had much higher average levels of isoflavones in urine (229 nmol/mg creatinine; median:145nmol/mg creatinine; range: 61–482nmol/mg creatinine) and plasma (1048.6 nmol/L; median: 663.1 nmol/L; range: 629.1–1853.6nmol/L).

For each group, the authors also calculated a body weight- and time-adjusted urinary isoflavone excretion (UIE) value that was further adjusted for dose per kg body weight [$(nmol \cdot h^{-1} \cdot kg^{-1})$/ $(mg \cdot kg^{-1} \cdot d^{-1})$]. The authors consider UIE expressed relative to time as more accurate than expressed relative to creatinine because creatinine is largely dependent upon muscle and body composition differs markedly in adults and infants. Dose per body weight corrected UIE in breastfed infants was 18.4 percent of the maternal value (25.5±3.3 versus 136.5±16.3), a statistically significant difference. In contrast, the UIE/dose was 24% higher in tofu-fed infants (169.4±32.1 versus 136.5); however, this increase was not statistically significant.

Breast milk

Seven studies were identified that report isoflavone levels in breast milk and those studies are outlined in **Table 17**. The highest background concentrations of isoflavones were observed in milk from women eating vegan and vegetarian diets.

Total levels of isoflavones in breast milk of mothers on an omnivorous, vegetarian, or vegan diet were reported by the UK Committee-on-Toxicity *(3)*. No information was provided on the methodology used to measure isoflavone levels in breast milk. **[Levels of genistein, daidzein, and equol were not reported separately in the UK Committee-on-Toxicity report. CERHR was not able to obtain the original report prepared by the UK Ministry of Agriculture, Fisheries, and Food.]** Levels of isoflavones in breast milk were orders of magnitude lower than levels in soy formula, which were reported at 18–41 mg aglycone equivalents/L prepared formula in a UK Ministry of Agriculture, Fisheries, and Food survey *(5; 96)*.

Table 17. Isoflavone Levels in Human Breast Milk

Population	Isoflavone Levels, ng/ml Mean ± SD or Mean (Range)				Reference
	Genistein	Daidzein	Equol	Total Isoflavone	
Observational Studies					
UK, omnivorous (n=14)				**[1 (0–2)]**[a]	Friar and Walker (MAFF 1998 report) as cited in Mortensen et al., 2009 *(14)*
UK, vegetarian (n=14)				**[4 (1–10)]**[a]	
UK, vegan (n=11)				**[11 (2–32)]**[a]	
US, omnivorous (n=9)	1.6 ± 1.9	3.1 ± 1.8	1.1 ± 0.8	5.6 ± 4.4	Setchell et al., 1998 *(6)*
NZ, (n=11, 2 were vegetarians)	Below LOD (50 µg/L)				Irvine et al., 1998a, 1998b *(90; 91)*
Soy Challenge Studies					
US, baseline (n=1)	~1	~0		~1	Setchell et al., 1998 ***(6)***
US, 1-day after ingesting 10 g toasted soy nuts containing 30 mg total isoflavones (n=1)	~10.5	~16.5		~26.5	
US, baseline (n=7)	**[0.55]**[b,d]	**[0.78]**[b,d]		**[1.3 ± 0.6]**[b,d]	Franke et al., 2006 *(95)* as cited in Mortensen et al., 2009 *(14)*
US, after daily consumption of soy protein beverage (36.5g) for 2–4 days (n=7)	**[7.6]**[b,d]	**[10.8]**[b,d]		**[18.5 ± 5.0]**[b,d]	
US, after daily consumption of soy protein beverage (36.5 g containing 55 mg total isoflavones) for 2–4 days (n=7)	**[10.3]**[b,d]	**[14.6]**[b,d]		**[24.9]**[b,d]	Franke et al., 2006 *(95)*
US or Chinese, baseline (n=1)[c]	~0	~0		35[e,f]	Franke et al., 1998*(81)*[e], as cited in Mortensen et al., 2009 *(14)*
US or Chinese, 96 hours after ingestion of roasted soybeans, 5 g at time 0, 10g at 24-h, 20g at 72-h (n=1)[c]	**[~21.6]**[f]	**[~30.5]**[f]		**[52.1]**[f]	Franke et al., 1998a, 1998b *(81; 105)*

[a] Presented in µg aglycone/kg milk by *(3)*. CERHR converted to µg/L because the density of breast milk is similar to water (specific gravity of breast milk is 1.03).
[b] CERHR converted units from nmol/L: µg/L = nmol/L × 0.27024 (genistein) or 0.25424 (daidzein).
[c] The sample size is unclear, it is either 1 or 2. The same set of data appear to be presented in both *(81; 105)*, however *(105)* suggest n = 2 and *(81)* suggest n=1.
[d] Frank et al., 2006 *(95)* present total isoflavone in nmol/L and state that 60% of the total in mother's milk was daidzein. CERHR used this ratio to estimate the proportion of total as genistein and daidzein and these estimates of genistein and daidzein were used to estimate a total isoflavone concentration in µg/L.
[e] Baseline concentrations in breast milk are not explicitly reported. Figure 3 in the study suggest values prior to soy challenge were near zero; the text states that following the soy challenge, the mean total isoflavone concentration in breast milk was 0.2µmol/L [=200 nmol/L, or ~52.1 µg/L]. It is unclear which values were used to derive the baseline value of 35µg/L reported in Mortensen et al.*(14)*.
[f] Frank et al.*(81)* reported that the mean total isoflavone concentration in breast milk following the soy challenge was 200 nmol/L. In a subsequent publication, Franke et al.*(95)* reported that 60% of the total isoflavone in mother's milk was daidzein. CERHR used this ratio to estimate the proportion of total as genistein and daidzein and these estimates of genistein and daidzein were used to estimate a total isoflavone concentration in µg/L.

APPENDIX II
Chemistry, Use and Human Exposure

Setchell *et al.*, 1997, 1998 *(6; 84)* used a gas chromatography (GC)-MS method to compare isoflavone levels in soy formula and human milk and to measure isoflavone levels in human milk following soy food ingestion. In comparison to soy formula, levels of isoflavones (measured by GC/MS) were substantially lower in cow-milk formula (below detection limit, which was not reported) and in milk collected from 9 healthy, omnivorous, lactating women (mean ± SD=5.6 ± 4.4 µg/L) *(6)*. Equol was detected in 7 of 9 human-milk samples. A >10-fold increase in milk isoflavone levels was observed following ingestion of 30 mg isoflavones + conjugates in the form of 10 g toasted soy nuts. **[Figure 2 in the study report suggests that milk daidzein levels increased >40-fold and genistein levels increased >10-fold 1 day after the ingestion of soy nuts.]** The study authors noted that isoflavones occur predominantly as glucuronide conjugates in human milk.

Irvine *et al.*, 1998 *(90)* used an HPLC method to measure isoflavone levels in dairy-based formula and in human milk. Levels of genistein and daidzein were below the detection limit (0.05 mg/L [50 µg/L]) in human milk samples from 11 mothers and in the dairy-based formulas. **[Based on this level of detection, measurable levels of isoflavones would not be expected based on concentrations of genistein, daidzein, and total isoflavones reported in [3; 6].]**

Franke *et al.*, 1998a, 1998b *(81; 105)* published two analytical methods papers on the use of HPLC with diode-array ultraviolet scanning to measure isoflavones in human fluids, including breast milk. As part of this work, genistein and daidzein was measured in samples of breast milk collected before and after consumption of roasted soy beans (5g at time 0, 10g at 24-h, 20g at 72-h) in 1 or 2 women. **[The sample size is unclear. The same set of data appear to be presented in both [81; 105], however *(105)* suggest n=2 and *(81)* suggest n=1.]** Concentrations of genistein and daidzein were undetectable at baseline and increased to ~70 nmol/L **[~19 µg/L]** and ~56 nmol/L **[~14 µg/L]**, respectively, after the soy challenge. Franke *et al.*, 1998b *(105)* reported a lack of detectable isoflavones after extraction without enzymatic hydrolysis, suggesting that isoflavones in milk occur primarily as glucuronide and/or sulfate conjugates.

Adults (blood)

Genistein and daidzein are readily detected in blood, including in populations not characterized as having high isoflavone intake such as Asian or vegetarian populations. **Table 18** surveys studies that report blood levels of isoflavones in adults resulting from typical dietary exposures. The daidzein metabolite, equol, can also detected in blood, but has a lower frequency of detection than daidzein and only ~30-50% of individuals are considered to be equol "producers" *(106)*. Fewer studies include measurement of glycitein. If glycitein is measured in humans, it is generally following intentional dosing of subjects with a soy protein or isoflavone supplement.

Only one study that reported background blood-based measurements of isoflavones was identified for adults in the US *(107)*. A number of US studies reported blood levels of isoflavones following ingestion of soy-based foods or supplements and these are discussed in Chapter 2. While these studies may contain data on pre-dosing blood levels, they have limited use in characterizing background exposures isoflavones in the general population because the sample sizes are typically small and study protocols often instruct subjects to avoid ingestion of soy products during the experiment.

Table 18. Blood Levels of Total Isoflavones in Adults

Population	*n*	*Plasma or serum levels, nM (µg/L), mean*[a]				*Reference*
		Genistein	*Daidzein*	*Equol*	*Glycitein*	
United States						
US men and women who participated in NHANES III, 1988–1994	209	17.4 (4.7)	15.3 (3.9)	< LOD		Valentín-Blasini et al., 2003 *(107)*
UK and Europe						
Finland, men, omnivore	14	0.5 (0.14)	0.6 (0.15)	0.1 (0.024)		Adlercreutz et al., 1993 as cited in Whitten and Patisaul, 2001 *(108)*
Finland, women, omnivore	14	4.9 (1.3)	4.2 (1.1)	0.8 (0.19)		Adlercreutz et al., 1994 *(109)*
Finland, men	14	6.3 (1.7)	6.2 (1.6)	0.8 (0.19)		Adlercreutz et al., 1993 *(110)*
Finland, women, omnivore[b]		7.7 (2.0)	6.4 (1.6)	1.6 (0.39)		Adlercreutz et al., 1993 *(111)*
Finland, women, vegetarian	14	17.1 (4.6)	18.5 (4.7)	0.7 (0.17)		Adlercreutz et al., 1994 *(109)*
Finland, women, lacto vegetarian	3	29.7 (8.0)	41.5 (11)	1.0 (0.059)		
Finland, women, vegetarian[b]		44.8 (12)	50 (13)	1.5 (0.36)		Adlercreutz et al., 1993 *(111)*
Portugal, men	50	Not Reported	1.3 (0.33)	0.35 (0.085)		Morton et al., 1997 as cited in Whitten and Patisaul, 2001 *(108)*
Britain, men	36	Not Reported	8.2 (2.08)	0.57 (0.138)		
Britain, women	80	30.1 (8.1)	13.5 (3.4)	Not Reported		Pumford et al., 2002 *(112)*
Britain, men	50	34.1 (9.2)	18.2 (4.6)	Not Reported		
Europe, women	718	6.3 (1.70)	3.1 (0.80)	0.62 (0.15)	0.46 (0.13)	Peeters et al., 2007 *(113)*
Europe, men	696	6.5 (1.77)	3.5 (0.89)	0.62 (0.15)	0.49 (0.14)	
Europe, men and women	1414	6.4 (1.73)	3.3 (0.84)	0.62 (0.15)	0.46 (0.13)	
Sweden, men and women from 2 cities: Umea and Malmo	173	2.6–4.1 (0.7–1.1)	2 (0.5)	0.8 (0.2)	0.4 (0.1)	
Denmark, men and women	100	4.4 (1.2)	4.3 (1.1)	0.4 (0.1)	0.4 (0.1)	
Netherlands, men and women	94	22.6 (6.1)	15.3 (3.9)	0.8 (0.2)	0.8 (0.2)	
Germany, men and women from 2 cities: Potsdam and Heidelberg	178	5.9–10.4 (1.6–2.8)	3.5–3.9 (0.9–1.0)	0.8 (0.2)	0.4 (0.1)	

Table 18 (continued)

Population	*n*	*Plasma or serum levels, nM (µg/L), mean*[a]				*Reference*
		Genistein	*Daidzein*	*Equol*	*Glycitein*	
France, women	41	6.7 (1.8)	2.8 (0.7)	0.8 (0.2)	0.4 (0.1)	Peeters et al., 2007 *(113)*
Italy, men and women from 3 cities: Varese/Turin, Florence, Ragusa/Naples	290	2.7–4.8 (0.7–1.1)	1.2–2.4 (0.3–0.6)	0.8 (0.2)	0.4 (0.1)	
Spain, men and women from 3 locations: Norther Spain, Murcia, and Granada	286	4.1–4.8 (1.1–1.3)	2–2.4 (0.5–0.6)	0.4 (0.1)	0.4 (0.1)	
Greece, men and women	87	2.7 (0.7)	1.2 (0.3)	0.4 (0.1)	0.4 (0.1)	
UK, men and women from Cambridge	95	15.5 (4.2)	8.3 (2.1)	0.4 (0.1)	0.8 (0.2)	
UK, men and women from Oxford, vegans and vegetarians	70	148 (40)	78.7 (20)	1.2 (0.3)	3.7 (0.9)	
Asia						
Japan, men consuming traditional Japanese diet	6	90–1204 (24–325)	60–924 (15–235)	0.54–24.6 (0.13–6.0)		Adlercreutz et al., 1994 *(109)*
Japan, men, omnivore	14	276.0 (75)	107.0 (27)	5.5 (1.3)		Adlercreutz et al., 1993 *(110)*
Japan, men, omnivore[c]		206.1 (56)	72.5 (18)	Not Reported		Arai et al., 2000 as cited in Whitten and Patisaul, 2001 *(108)*
Japan, men[a]	101	493.3 (133)	280.7 (71)	Not Reported		Pumford et al., 2002 *(112)*
Japan, women[a]	125	501.9(136)	246.6 (63)	Not Reported		
Japan, women	106	307.5 (83)	111.7 (28)	Not Reported		Arai et al., 2000 *(114)*
Hong Kong, men	53	Not Reported	31.3 (8)	3.8 (0.9)		Morton et al., 1997 as cited in Whitten and Patisaul, 2001 *(108)*

[a] 1 nM = 0.270 µg/L or 270.24 ng/L genistein, 0.254 µg/L or 254.24 ng/L daidzein, 0.284 µg/L or 284.26 ng/L equol, and 0.284 µg/L or 284.16 ng/L glycitein. Conversions in the table refer to aglycone equivalents.

[b] **[Sample size unclear, article references 27 pre and postmenopausal omnivorous and vegetarian women].**

[c] **[Sample size unclear].**

Table 19. Comparison of Urinary and Serum Concentrations of Isoflavones from a Non-Representative Sample of Adults who Participated in NHANES III, 1988–1994

Isoflavone or Metabolite	Mean Concentration, ng/ml	
	Urine (n = 199)	Serum (n = 209)
Genistein	129	4.7
Daidzein	317	3.9
Equol	36	< LOD
O-DMA	50	1.0

From Valentín-Blasini et al., 2003 *(107)*.

Valentín-Blasini *et al.*, 2003 *(107)* measured serum levels of genistein, daidzein, equol, and *O*-desmethylangolensin from 209 samples taken from a nonrepresentative sample of adults who participated in NHANES III, 1988-1994 (see **Table 19**). Samples were analyzed by solid-phase extraction (SPE) followed by HPLC and tandem mass spectrometry and were hydrolyzed by the addition of b-glucuronidase/sulfatase. In serum, the concentrations of genistein were the highest (4.7 ng/ml, range of n.d.–203 ng/ml), followed by daidzein (3.9 ng/ml, range of n.d.–162 ng/ml), *O*-DMA (1.0 ng./ml, range of n.d.–29 ng/ml), and equol (< LOD, range of n.d.–8.2 ng/ml). **[The authors noted that levels of equol reported by other investigators were below the limit of detection in this study, described as being in the "low" ng/ml range]**. Serum levels were several times lower than the corresponding urine measurements. Highly significant correlations between levels in urine and serum samples from the same persons were observed for genistein (r=0.79, *P*=0.0001), daidzein (r=0.72, *P*=0.0001), and *O*-desmethylangolensin (r=0.41, *P*=0.0007).

In Finland and Canada, genistein blood concentrations were reported at 0.5–8 nM **[0.14–2.16 µg/L aglycone equivalent]** in omnivores and 17–45 nM **[4.6–12 µg/L aglycone equivalent]** in vegetarians.

Mean plasma levels of isoflavones in infants fed soy formulas were ~5–20 times higher than Japanese adults or adults ingesting similar levels of total isoflavones from soy-based foods ~90–1200 nM **[24–324 µg/L aglycone equivalent]**), ~50 times higher than vegetarian adults in Western populations, and ~500 times higher than omnivorous adults in Western populations *(6; 84)*. Dietary exposure estimates comparing total isoflavone intake in infants ingesting soy formula versus adults are fairly consistent to comparisons based on plasma total isoflavone levels.

Adults (urine)

In July 2008, the U.S. Centers for Disease Control and Prevention (CDC) released the "National Report on Biochemical Indicators of Diet and Nutrition in the U.S. Population 1999-2002" *(115)*. This report presents information on urinary concentrations of genistein, daidzein, and equol in ~5350 Americans age 6 years and older who took part in CDC's National Health and Nutrition Examination Survey (NHANES) during all or part of the four-year period from 1999 through 2002. Results are summarized in **Table 20.** **[The Expert Panel noted several points regarding the data presented for NHANES 1999–2002. Biomonitoring data have been used to estimate prevalence and magnitude of exposure to isoflavones but not to estimate isoflavone intake. Genistein was not measured in children younger than 6 years of age, but it is very likely that genistein would be detected in that age group. Genistein measurements were not separately reported for Asian Americans because of the comparatively small group size.**

Table 20. Geometric Mean and Selected Percentiles of Urine Concentrations for the Total U.S. Population Aged 6 years and Older, NHANES, 1999–2002

Population	Isoflavones, Geometric Mean (10^{th}-90^{th} percentile)								
	Genistein			Daidzein			Equol		
	n	µg/L	µg/g creatinine	n	µg/L	µg/g creatinine	n	µg/L	µg/g creatinine
Age group (years)									
6–11	721	33.8 (5.80–248)	36.7 (6.94–243)	726	88.1 (15.6–544)	95.6 (18.4–635)	668	11.5 (< LOD-45.9)	12.4 (< LOD-45.6)
12–19	1498	38.0 (5.60–314)	27.5 (4.36–192)	1497	89.7 (10.9–786)	64.8 (8.03–600)	1401	10.4 (< LOD-43.1)	7.68 (< LOD-30.3)
20–39	1140	31.3 (3.10–349)	25.1 (3.77–244)	1138	63.7 (6.59–590)	51.1 (5.91–523)	1043	8.54 (< LOD-37.6)	6.90 (< LOD-30.3)
40–59	951	23.7 (2.22–298)	23.8 (2.48–270)	951	52.3 (5.62–505)	52.6 (5.54–535)	909	8.39 (< LOD-37.1)	8.40 (< LOD-37.3)
60+	1035	24.1 (2.90–227)	28.9 (4.48–222)	1035	44.8 (4.80–394)	53.7 (6.01–389)	955	7.52 (< LOD-34.5)	9.02 (< LOD-37.8)
Sex									
Male	2597	31.1 (3.86–298)	25.0 (4.02–203)	2595	64.8 (7.29–542)	52.1 (5.96–497)	2417	9.26 (< LOD-39.6)	7.39 (< LOD-31.1)
Female	2754	26.1 (2.85–285)	28.1 (3.50–265)	2752	57.5 (6.41–553)	61.9 (7.18–564)	2559	8.33 (< LOD-37.6)	9.10 (< LOD-37.8)
Race/Ethnicity									
Mexican American	1498	29.7 (4.07–294)	27.6 (3.85–243)	1495	55.9 (5.10–618)	52.0 (4.81–546)	1405	6.16 (< LOD-25.0)	5.77 (< LOD-26.3)
Non-Hispanic, Black	1289	31.5 (3.73–328)	21.2 (3.24–192)	1288	76.1 (8.10–628)	51.3 (5.54–447)	1196	6.89 (< LOD-28.8)	4.68 (< LOD-33.6)
Non-Hispanic, White	2112	27.1 (2.96–281)	27.0 (3.80–242)	2112	58.4 (6.60–505)	58.1 (6.90–506)	1955	9.62 (< LOD-41.3)	9.61 (< LOD-38.7)
Total	5351	28.5 (3.27–293)	26.6 (3.78–242)	5347	61 (6.75–545)	56.9 (6.67–536)	4976	8.77 (< LOD-38.5)	8.22 (< LOD-35.1)

LOD = Limit of detection

From CDC 2008 National Report on Biochemical Indicators of Diet and Nutrition in the U.S. Population 1999–2002 *(115)*.

It is possible that Asian Americans consume more genistein-containing products than other races/ethnicities in the US. It is not possible to determine regional/geographical variations from the NHANES data. Total (conjugated + free) concentrations of genistein were measured using high performance liquid chromatography coupled to isotope dilution tandem MS (HPLC-MS/MS).]

The CDC made several observations based on the uncorrected tables of 1999–2002 data contained in the report. These observations between demographic groups noted below were based on non-overlapping confidence limits from univariate analysis without adjusting for demographic variables (i.e., age, sex, race/ethnicity) or other determinants of these urine concentrations (i.e., dietary intake, supplement usage, smoking, BMI). The CDC noted that multivariate analysis may alter the size and statistical significance of these categorical differences. Also, additional significant differences of smaller magnitude may be present if confidence limits slightly overlap or if differences are not statistically significant before covariate adjustment has occurred.

- Urinary isoflavone (genistein, daidzein, equol, and ODMA) concentrations are generally lower in adults than they are in children and adolescents.
- Males and females have similar phytoestrogen concentrations.
- Non-Hispanic whites have higher equol concentrations than non-Hispanic blacks and Mexican Americans. Mexican Americans have lower ODMA concentrations than non-Hispanic blacks and non-Hispanic whites.

Overall, the CDC concluded that urinary isoflavone concentrations show only small variations by demographic variables such as age, sex, or race/ethnicity. The Third National Report on Human Exposure to Environmental Chemicals also looks at the urinary phytoestrogens for the same period but in separate two-year periods (1999-2000 and 2001-2002) *(116)*. Least squares adjusted geometric means were compared, adjusted for covariates of race/ethnicity, age, sex and urinary creatinine. Some of the observations made were:

- "In NHANES 2001-2002, both urinary genistein and daizein levels were higher in the group aged 6-11 years than in either of the groups aged 12-19 years or 20 years and older, and females had higher levels than males. One study found that levels were higher in males for all phytoestrogens except equol (Lampe *et al.*, 1999)."
- "In NHANES 2001-2002, adjusted geometric mean levels of urinary equol were higher for non-Hispanic whites than for Mexican Americans or non-Hispanic blacks. Adjusted geometric mean levels of urinary equol were higher in the group aged 6-11 years than in either of the other two age groups."
- "In NHANES 2001-2002, adjusted geometric mean levels of urinary O-desmethylangolensin were higher in non-Hispanic whites than in Mexican Americans. The group aged 20 years and older had lower adjusted geometric mean levels of urinary O-desmethylangolensin than either of the other two age groups."
- "In NHANES 2001-2002, both urinary enterodiol and enterolactone levels were higher in the group aged 6-11 years than in the group aged 12-19 years. Levels of the lignans previously have been reported to differ by race (Horn-Ross *et al.*, 1997), and in an NHANES III statistical analysis, to differ by income, gender, and age (Valentin-Blasini *et al.*, 2003)."

[The Exposure Report does not make any comparisons with the 1999-2000 data, nor does it state a reason for this. The comparisons made seem to be consistent with the observations in the 1999-2002 set used in the National Report on Biochemical Indicators of Diet and Nutrition.]

A previous CDC assessment of isoflavone concentrations in ~2500 urine samples from individuals who participated in the NHANES in 1999 and 2000 was published by Valentín-Blasini *et al.*, 2005 *(117)*. Phytoestrogens were detected in over 70% of the samples analyzed; enterolactone was detected in the highest concentrations, and daidzein was detected with the highest frequency. This publication also included a summary of daily urinary excretion rates of genistein, daidzein, equol and *O*-DMA reported in different studies for various populations. Valentín-Blasini *et al.*, 2005 *(117)* concluded that, in general, the reported concentrations were similar to those in US adults with non-supplemented diets. However, populations with isoflavone enriched diets, whether by food or dietary supplements, had much higher isoflavone concentrations, up to two orders of magnitude higher. In addition, Asian populations typically had much higher concentrations as well. **Table 21** summarizes daily urinary excretion rates of genistein, daidzein, and equol based on the most recent CDC report *(115)*, studies summarized in Valentín-Blasini *et al.*, 2005 *(117)*, and new literature published since the initial NTP-CERHR Expert Panel evaluation of soy formula *(17)*

1.2.2.7 Estimated Isoflavone Intake in Adults

A relatively large number of studies have published estimates of isoflavone intake and a survey of those studies is presented in Tables 22-25, with an emphasis on studies with large sample sizes that represent typical consumption in Western and Asian countries or intakes in specific populations of interest, e.g., vegetarians.

In the US, estimates of isoflavone intake based on samples that are designed to be representative of the general population range from 0.68 mg/day *(66)* to 1.2 mg/day *(121)*. The estimate of 0.68 mg/day of total isoflavones (**Table 22**) is based on work conducted by researchers at the USDA who also estimated dietary intake of genistein (0.33 mg/day), daidzein (0.31 mg/day), and glycitein (0.03 mg/day) in the U.S. diet by combining data from the 2008 USDA Database for the Isoflavone Content of Selected Foods, the FNDDS, and the WWEIA, which is the dietary intake interview component of NHANES (Haytowitz *et al.*, 2009 *(66)*, discussed in more detail in **Section 1.2.2.4**).

Table 22. Per Capita Consumption of Isoflavones in the U.S. Diet for All Ages

Isoflavone	*Consumption, mg/day*
Total isoflavones	0.68
Genistein	0.33
Daidzein	0.31
Glycitein	0.03

From *http://www.ars.usda.gov/SP2UserFiles/Place/12354500/Articles/EB09_Isoflavone.pdf*.

Chun *et al.*, 2007 also used the USDA isoflavone database and NHANES dietary recall data to estimate intakes (**Table 23**). Through a series of steps, Chun *et al.*, 2007 converted the 1999-2002 NHANES dietary recall data to USDA Standard Reference codes and linked them with the 2002 USDA database (referred to by the authors as the USDA Flavonoid Database, or FLDB).

Table 21. Daily Urinary Excretion of Isoflavones in Adults

Population	n	Mean Total Urinary Isoflavone, nmol/day (10^{th} – 90^{th} percentile)			References
		Genistein	Daidzein	Equol	
US and Canada					
US, general population, ages 6+	5351	211 (24–2,171)	480 (53–4,287)	72 (< LOD-318)	US Centers for Disease Control and Prevention, 2008 *(115)*
US, women providing multiple 24-hour urine samples or overnight urine samples	27	1,083–1,990[a]	2,689–3,601[a]	753–1,098[a]	Tseng et al., 2008 *(68)*
US, adult women ingesting diet with 2.01 mg/kg bw/day isoflavones	11	14,200[c]	9,528[c]	438[c]	Xu et al., 1998 *(118)*
US, adult women ingesting diet with 1.01 mg/kg bw/day isoflavones	11	6,529[c]	4,964[c]	270[c]	Xu et al., 1998 *(118)*
US, tofu-dosed volunteers (> 1/week)	7	2,515[d]	4,320[d]	2,625[d]	Franke 1994 as cited in Valentín-Blasini et al., 2005 *(117)*[b]
US, adult men ingesting tempeh diet	17	1,719	3,630	441	Hutchins et al., 1995*(119)*
US, adult men ingesting soy diet	17	1,658	3,875	718	Hutchins et al., 1995*(119)*
US, studies of adults ingesting high soy diets	20	1,410	22,880	1,090	Kirkman et al., 1995 as cited in Valentín-Blasini et al., 2005 *(117)*[b]
US, tofu-dosed volunteers (< 1/week)	16	307[d]	475[d]	643[d]	Franke et al., 1994 as cited in Valentín-Blasini et al., 2005 *(117)*
Europe					
Finland, women, omnivorous	1		180	380	Bannwart et al., 1984 as cited in Valentín-Blasini et al., 2005 *(117)*
Finland, women, vegetarian	4		3,290[d]	34,770[d]	Bannwart et al., 1984 as cited in Valentín-Blasini et al., 2005 *(117)*
Germany, men on normal diet (age range 18–32)	**7**	333.33–1770.4[a]			Moors et al., 2007 *(120)*
Italy, postmenopausal women taking placebo in soy supplement study	29	844[d,e]	1,764[d,e]	350[d,e]	Albertazzi et al., 1999 as cited in Valentín-Blasini et al., 2005 *(117)*[b]
Italy, postmenopausal women taking soy supplement	29	20,874[d,e]	23,275[d,e]	8,250[d,e]	Albertazzi et al., 1999 as cited in Valentín-Blasini et al., 2005 *(117)*[b]
Netherlands, postmenopausal women with and without breast cancer	100 with cancer 100 without cancer	1,519–1,746[e]			Den Tonkelaar et al., 2001 as cited in Valentín-Blasini et al., 2005 *(117)*

Table 21 (continued)

Population	*n*	*Mean Total Urinary Isoflavone, nmol/day (10^{th} – 90^{th} percentile)*			*References*
		Genistein	*Daidzein*	*Equol*	
Asia					
China, postmenopausal women		1,470	3,240		Roach et al., 1998 as cited in Valentín-Blasini et al., 2005 *(117)*
China, adult women with and without breast cancer	250 with cancer 250 without cancer	14,264–17,246[d]	27,134–37,044		Dai et al., 2002 as cited in Valentín-Blasini et al., 2005 *(117)*
Japan, men and women	2 men 4 women	1,769–6,476[d]	4,303–14,255	156–298	Adlercreutz et al., 1995a as cited in Valentín-Blasini et al., 2005 *(117)*
Japan, adult women	105	10,790[f]	20,540[f]	15,740[f]	Arai et al., 2000 *(114)*
Japan, adult women with documented intakes of isoflavones	111	10,000	25,000		Uehar et al., 2000 as cited in Valentín-Blasini et al., 2005 *(117)*
Korea, postmenopausal women: Control, with osteopenia, with osteoporosis	25 controls 29 with osteopenia 21 with osteoporosis	225–384[d,f]	616–852[d,f]	109–137[d,f]	Kim et al., 2002 and Dai et al., 2002 as cited in Valentín-Blasini et al., 2005 *(117)*

[a] Calculated by CERHR by assuming 2.145 g creatine excreted/day and converting µg to nmol.
[b] See Valentín-Blasini et al., 2005 *(117)* for complete reference.
[c] The values were obtained from the primary study report because the values provided by Valentín-Blasini et al., 2005 *(117)* appeared to be in error.
[d] Study authors calculated values by assuming 2.145 g creatinine excreted/day or 2000 mL urine/day.
[e] Study authors assumed that daidzin and genistin measured in urine actually referred to the aglycones.
[f] Median values.
To convert nmol to genistein equivalents in µg, multiply by 0.27.
Adapted from Valentín-Blasini et al., 2005 *(117)*.

Table 23. Total Isoflavone Intake Based on in Adult Participants in NHANES 1999–2001 who Completed the 24-hour Dietary Recall and Food Frequency Questionnaire

Population		*n*	*Isoflavone Intake, mg/day (mean ± SD)*
All		8809	1.2 ± 0.2
Sex			
Men		4461	1.1 ± 0.2
Women		4348	1.3 ± 0.2
Age			
19–30		1873	1.2 ± 0.4
31–50		2835	1.3 ± 0.2
51–70		2582	1.2 ± 0.2
70+		1519	0.9 ± 0.2
Ethnicity			
Non-Hispanic white		4512	1.2 ± 0.2
Non-Hispanic black		1762	1.2 ± 0.3
Mexican-American		2141	0.8 ± 0.2
Others		694	1.7 ± 0.4
Poverty-Income Ratio[a]			
< 1.0		1503	0.5 ± 0.1
1.0–1.3		820	0.8 ± 0.4
1.3–1.85		1078	0.5 ± 0.1
≥ 1.85		4496	1.5 ± 0.2
Current Smoking			
No		4247	1.5 ± 0.3
Yes		4088	0.9 ± 0.1
Vitamin Supplement Use			
No		4994	0.8 ± 0.2
Yes		3719	1.6 ± 0.3
Food Group (based on 24-h dietary recall)[b]			
Fruits and fruit juices	Non-consumers	2963	0.6 ± 0.1
	Consumers (3rd tertile, > 300.8 g/d)	1947	2.0 ± 0.3
Vegetables and vegetable products	Non-consumers	729	0.8 ± 0.4
	Consumers (3rd tertile, > 261/1 g/d)	2691	1.7 ± 0.4
Wine	Non-consumers	8159	1.1 ± 0.2
	Consumers (3rd tertile, > 206.5 g/d)	207	2.2 ± 1.0
Tea	Non-consumers	6934	1.1 ± 0.1
	Consumers (3rd tertile, > 606.8 g/d)	621	2.3 ± 0.8

[a] Poverty income ratio = ratio of median family income over the poverty index; a value of ≤ 1.30 is required for eligibility in food assistance programs.
[b] All subjects who did.not consume the food in the 1-d 24-h dietary recall were grouped as non-consumers.
From Chun et al., 2007 *(121)*.

Intake was based on 45% of all foods recalled in NHANES 1999–2002. Isoflavones contributed 0.6% to the total intake of flavonoids. There appeared to differences in intake based on age, ethnicity, income, and use of alcohol and vitamin supplements. There were no apparent differences based on alcohol use or non-leisure time physical activity level. Chun *et al.*, 2007 noted a number of limitations to the estimated intakes, including the exclusion of intakes from herbal and isoflavone supplements.

It is not clear why the estimates of total isoflavone consumption of 0.68 mg/day calculated by Haytowitz *et al.*, 2009 *(66)* is lower than the estimate of 1.2 mg/day presented by Chun *et al.*, 2007 *(121)*. Both estimates are based on use of at least some of the same databases. Differences in intake estimates could reflect use of different versions of various databases, i.e., Hawtowitz used the 2008 USDA isoflavone, 2006 FNDD, and 2005-2006 NHANES data files while Chun used 2002 USDA isoflavone and 1999-2000 NHANES data. However, the use of more recent databases by Haytowitz *et al.*, 2009 *(66)* might be expected to result in higher estimated intakes given the rise in popularity of soy foods and products. Other differences in methodology as well as assumption used to fill fields with missing values in the USDA database, e.g., zero or another value based on other information may contribute to the differences in estimated intakes.

In 2009, Chun *et al. (122)* applied a similar strategy to that used in the 2007 publication on 2,908 US adults in the 1999-2002 NHANES for which both dietary recalls and urinary isoflavone concentrations were available to assess the validity of estimating dietary intake based on urinary concentrations. Daily isoflavone intake was reported by 35% of subjects. Total isoflavone intake averaged 1.0 ± 0.2 mg/1,000 kcal/day for the total sample (**Table 24**) and 3.1 ± 0.7 mg/1,000 kcal/day for those who consumed isoflavones. Genistein was the major contributor (55%) to total daily isoflavone intake, followed by daidzein (35%), glycitein (7%), biochanin A (1%), and formononetin (1%). Chun *et al.*, 2009 *(122)* also reported statistically significant, dose-dependent associations between total urinary concentrations with overall dietary isoflavone intake regardless of dietary isoflavone source, but no association was detected between the overall isoflavone intake and urinary concentrations of equol.

Table 24. Isoflavone Intake based on in Adult Participants in NHANES 1999–2001 for which Dietary Recall and Urinary Isoflavone Concentrations were Available

Population	*n*	*Isoflavone Intake, mg/1,000 kcal/day (mean ± SD)*			
		Genistein	*Daidzein*	*Glycitein*	*Total*[a]
All	2,908	0.6 ± 0.1	0.4 ± 0.1	0.1 ± 0.0	1.0 ± 0.2
Sex					
Male	1,495	0.6 ± 0.2	0.4 ± 0.2	0.1 ± 0.0	1.1 ± 0.4
Female	1,413	0.6 ± 0.1	0.4 ± 0.1	0.1 ± 0.0	1.0 ± 0.2
Age					
19–29	585	0.7 ± 0.5	0.4 ± 0.3	0.1 ± 0.1	1.2 ± 0.9
40–49	528	0.9 ± 0.3	0.6 ± 0.2	0.1 ± 0.0	1.7 ± 0.5
≥70	535	0.3 ± 0.1	0.2 ± 0.1	0.0 ± 0.0	0.6 ± 0.2

[a]Total isoflavone intake is based on the sum of genistein, daidzein, glycitein, biochanin A and formononetin.
From Chun et al., 2009 *(122)*.

One of the studies reported values for vegetarians residing in the UK. The review by the UK Committee on Toxicity *(3)* reported total isoflavone intakes of up to 150 mg/day in vegans, a value that is about an order of magnitude higher than maximum isoflavone intakes for other populations listed in **Table 25**.

Several studies reporting genistein and daidzein aglycone + glycoside intakes in Asian populations were included in **Table 25** because the values may compare to intakes by Asian Americans consuming their traditional diets. Asian Americans consuming traditional diets are likely to be a subpopulation among the most highly exposed to genistein and its conjugates. Estimates of aglycone + conjugated genistein and isoflavone intake within all population groups are highly variable. **[While these estimates cover a wide range, there are clues to suggest that the divergent values are not artifacts of different methodology. For example, two studies of vegetarian intake [3; 123] yield similar intake estimate despite using different methods to estimate intake: questionnaires and analytical measurement. In one of these papers *(123)* questionnaires were used to study omnivores, yielding intake estimates 10–100-fold higher than those from two other questionnaire studies [124; 125], which assessed older populations. Higher intake estimates in populations of Asian people may be attributable to diets including more soy products.]**

A recent review by Mortensen *et al.*, 2009 *(14)* reports the estimated isoflavone intake in adults from 15 countries, including 2 studies evaluating vegetarians, vegans and soy-consumers in European countries. Mortensen *et al.*, 2009 *(14)* observed that a traditional Asian diet leads to a mean daily isoflavone intake of ~8-50 mg in Asian populations with the intake of older Japanese adults ranging from 25-50 mg/day. The estimated Korean intake is ~14.9 mg/day and the mean isoflavone intake of women in China is reported in the range of ~7.8-61 mg/day and differs depending on the region in China. Intakes of isoflavone in Western populations are lower than in Asian populations, mainly a result of differences in diet. In addition, Mortensen *et al.*, 2009 report that the intake of vegetarians and soy-consumers (3-12 mg/day) is lower than the estimated intake in Asian population (15-60 mg/day); however the estimated isoflavone intake of vegan breast-feeding mothers in the UK (75 mg/day) is higher than the Asian population.

Validation of food intake questionnaires

Most of the studies cited in **Table 25** rely on food frequency questionnaires (FFQ) to estimate isoflavone intake. A number of studies have tried to assess the degree of correlation between reported isoflavone intakes from a questionnaire with a biologically-based indicator of exposure, typically urinary isoflavone concentrations. French *et al.*, 2007 *(67)* reported that recent, i.e., previous 2 days, dietary isoflavone intake significantly correlates with urinary excretion of metabolites ($r=0.64$ $P<0.001$), as does habitual (previous 2 months) dietary intake (correlation value $r=0.54$, $P=0.004$).). In this case, the dietary assessment was done by food frequency questionnaire containing 53 items completed during an interviewer-assisted interview.

Lampe *et al.*, 1999, 2003 *(57; 58)* examined the cross-sectional association between urinary isoflavonoid and lignan excretion and intakes of vegetables and fruits in a healthy adult population in the US (49 males and 49 females; 18–37 years old, 91% Caucasian). Dietary intakes were assessed using 5-day diet records and a food frequency questionnaire. Vegetable and fruit intake groupings (total vegetable and fruit, total vegetable, total fruit, soy foods, and vegetable and fruit grouped by botanical families) were used to assess the relationship between vegetable and fruit intake and urinary isoflavonoid and lignan excretion.

Table 25. Estimated Isoflavone Intakes in Adults

Population	*n*	*General Method of Estimate*	*Mean Intake, mg aglycone equivalents/day (median)*			*Reference*
			Genistein	*Daidzein*	*Total Isoflavone*	
United States and Canada						
US men, mean age 60.6 years old	107	FFQ DietSys database	(0.0297) range 0–0.947	(0.0228) range 0–20.95	**[0.0525]**[b] **[range 0–21.9]**[b]	Strom et al., 1999 *(125)*
US men with prostate cancer, mean age 61 years old	83	FFQ DietSys database	(0.0198) **[range 0–0.970]**	(0.0142) **[range 0–4.38]**	**[0.034]**[b] **[(range 0–5.35)]**[b]	Strom et al., 1999 *(125)*
US per capita consumption, both sexes and all ages		Combining data from the 2008 USDA Database for the Isoflavone Content of Selected Foods, the FNDDS, and the WWEIA	0.33	0.31	0.68	Haytowitz et al., 2009 *(66)*
US postmenopausal Caucasian women who participated in the Framingham Offspring Study, ages not reported	964	FFQ IF levels based on scientific literature and consultation with experts	0.338 (0.070)	0.289 (0.039)	0.760[a] (0.154)[a]	de Kleijn et al., 2001*(124)*
US men and women	8,809	Combining data from the 2002USDA Database 1999–2002 NHANES dietary recall data			1.2	Chun et al., 2007 *(121)*
US women, sub-study of the California Teachers Study cohort, < 85 years old,	185	FFQ IF levels based on database developed by authors	(0.7) range 0.1–38.9	(0.7) range < 0.1–31.2	1.5[a] range 0.2–70.2[a]	Horn-Ross et al., 2006 *(126)*
US women, 50–79 years old	447	FFQ IF levels based on database developed by authors			2.87	Horn-Ross et al., 2000 as cited in Mortensen et al., 2009 *(14)*
US women, 35–79 years old	2,882	FFQ IF levels based on database developed by authors			3.3	Horn-Ross et al., 2001 as cited in Mortensen et al., 2009 *(14)*
US women in Family Risk Assessment Program	451	Harvard Diet Assessment Form (DAF) and a soy food questionnaire developed for non-Asian women (SFQ);	2.4–3.9	2.3–2.8	**[4.7–6.7]**[b]	Tseng et a. 2008 *(68)*

Table 25 (continued)

Population	*n*	*General Method of Estimate*	*Mean Intake, mg aglycone equivalents/day (median)*			*Reference*
			Genistein	*Daidzein*	*Total Isoflavone*	
US omnivorous or semi-vegetarian men and women, 20–69 years old	29	FFQ, IF levels based on scientific literature	192 mg/month **[6 mg/day]**	110 mg/month **[4 mg/day]**	**[10 mg/day]**[b]	Kirk et al., 1999 *(123)*
Canadian women, 11% self-identified vegetarians, 25–42 years old	26	FFQ IF levels based on scientific literature			13.7	French et al., 2007 *(67)*
US vegetarian men and women, 20–69 years old	22	FFQ IF levels based on scientific literature	297 mg/month **[10 mg/day]**	158 mg/month **[5 mg/day]**	**[15 mg/day]**[b]	Kirk et al., 1999 *(123)*
Hawaiian women, 36–80 years old	102	FFQ IF levels based on USDA Nutrient Database for soy protein and scientific literature			5.2–18.9	Maskarinec et al., 1998 *(127)*
Filipino					5.2	
Caucasian					5.2	
Chinese					11.9	
Hawaiian					12.1	
Other					16.8	
Japanese					18.9	
British Isles						
UK men and women, 40–64 years old	335	7-day dietary record IF levels based on VENUS database			0.70	Van Erp-Baart et al., 2003 as cited in Mortensen et al., 2009 *(14)*
UK men, 19–76 years old	Men: 1 Women: 8	7-day dietary record IF levels based on database constructed by authors			1.2	Ritchie et al., 2006 *(62)*
UK men, 50–74 years old	203	FFQ IF levels based on scientific literature			(1.0)	Heald et al., 2006 as cited in Mortensen et al., 2009 *(14)*
UK women, 25 -75 years old	50	Monthly 24 hour recalls for 1 year IF concentrations based on scientific literature			0.37	Bhakta et al., 2006 as cited in Mortensen et al., 2009 *(14)*

Population	*n*	*General Method of Estimate*	*Mean Intake, mg aglycone equivalents/day (median)*			*Reference*
			Genistein	*Daidzein*	*Total Isoflavone*	
Ireland, men and women, 18–64 years old	1,379	7-day dietary record IF levels based on VENUS database			0.73	Van Erp-Baart et al., 2003 as cited in Mortensen et al., 2009 *(14)*
Ireland, soy-consumers	42	7-day dietary record IF levels based on VENUS database			6.0	
UK vegetarians, numbers and ages of volunteers not specified		7-day duplicate diet analysis IF intake estimated from isoflavone concentrations in duplicate diet, weights of samples, and weights of study participants.	8	4	**[12 mg/day]**[b]	UK Committee on Toxicology 2003 *(3)*
UK vegetarian men, 21–56 years old	Men: 1 Women: 9	7-day dietary record IF levels based on database constructed by authors			7.4	Ritchie et al., 2006 *(62)*
UK vegetarians	35	7-day duplicate diet analysis			12	Clarke et al., 2003 as cited in Mortensen et al., 2009 *(14)*
UK vegan breast-feeding mothers	11	Duplicate diet analysis			75	Friar and Walker (MAFF 1998 report) as cited in Mortensen et al., 2009 *(14)*
UK soy-consumers	15	7-day record Concentration based on VENUS database			3.2	Van Erp-Baart et al., 2003 as cited in Mortensen et al., 2009 *(14)*
Europe						
(European Prospective Investigation into Cancer and Nutrition (EPIC) study), men and women, 35-74 years old Denmark, France, Germany, Greece, Italy, Norway, Spain, Sweden, Netherlands, UK	35,955	IF levels based on USDA-Iowa State University database			< 2	Keinan-Boker et al., 2002 as cited in Mortensen et al., 2009 *(14)*
Men who did not consume soy, part of EPIC	5602	7-day record IF levels based on in-house food composition database	0.35	0.29	**[0.64]**[b]	Mulligan et al., 2007 *(128)*

Table 25 (continued)

Population	*n*	*General Method of Estimate*	*Mean Intake, mg aglycone equivalents/day (median)*			*Reference*
			Genistein	*Daidzein*	*Total Isoflavone*	
Men who consume soy; part of EPIC	169	7-day record IF levels based on in-house food composition database	3.96	3.05	**[7.01]** [b]	Mulligan et al., 2007 *(128)*
Women who did not consume soy; part of EPIC	5870	7-day record IF levels based on in-house food composition database	0.27	0.22	**[0.49]** [b]	Mulligan et al., 2007 *(128)*
Women who consume soy; part of EPIC	202	7-day record IF levels based on in-house food composition database	4.37	3.48	**[7.85]** [b]	Mulligan et al., 2007 *(128)*
Netherlands, women, 50–69 years old	17,140	FFQ IF levels based on scientific literature			0.88	Keinan-Boker et al., 2002 as cited in Mortensen et al., 2009 *(14)*
Netherlands, soy-consumers	85	2-day dietary record IF levels based on VENUS database			11.1	Van Erp-Baart et al., 2003 as cited in Mortensen et al., 2009 *(14)*
Netherlands, men and women, 1–97 years old	4,085	2-day dietary record IF levels based on VENUS database			0.91	Van Erp-Baart et al., 2003 as cited in Mortensen et al., 2009 *(14)*
Switzerland, men and women	Missing n	IF levels calculated with data from soybeans used to produce food products (traditional soy-based foods not included)			1.7	Rupp et al., 2000 as cited in Mortensen et al., 2009 *(14)*
Finland, men and women, 24–64 years old	2,862	24-hour recall IF levels based on Finnish National Food Composition database			0.79	Valsta et al., 2003 as cited in Mortensen et al., 2009 *(14)*
Italy, men and women, ≤ 94 years old	1513	7-day dietary record IF levels based on VENUS database			0.55	Van Erp-Baart et al., 2003 as cited in Mortensen et al., 2009 *(14)*
Japan						
Japan, no information on study population		No details available	5.4–9.3			Fukutake et al., 1997 as cited in Fitzpatrick, 1998 *(129)*
Japan, women, 29–78 years	106	3-day dietary record IF levels based on levels reported in Japanese food e	30.2	16.4	46.6	Arai et al., 2000 *(114)*
Japan, men	Men: 886 Women: 346	1-day dietary record IF levels based on scientific literature	(19.6)	(12.1)	[(31.7)] [b]	Wakai et al., 1999 *(130)*

Table 25 (continued)

Population	*n*	*General Method of Estimate*	*Mean Intake, mg aglycone equivalents/day (median)*			*Reference*
			Genistein	*Daidzein*	*Total Isoflavone*	
Japan, men	Men: 46 Women: 42	Four 4-day dietary records during a year (food and beverage portions weighed when possible) IF levels based on scientific literature	(14.9)	(9.5)	**[(24.4)]**[b]	Wakai et al., 1999 *(130)*
Japan, women, mean age 42.5 years	201	FFQ			29.9	Nagata et al., 2003 as cited in Messina et al., 2006 *(131)*
Japan, women, mean age 50.9 years	206	FFQ			26.0	Takata et al., 2004 as cited in Messina et al., 2006 *(131)*
Japan, women, 19–34 years old	189	FFQ			26.9	Nagata et al., 2004 as cited in Messina et al., 2006 *(131)*
Japan, women, 44–80 years old	478	Dietary interview			54.3	Somekawa et al., 2001 as cited in Messina et al., 2006 *(131)*
Japan, women, mean age 42.9 years	1,172	FFQ			37.4	Nagata et al., 2001 as cited in Messina et al., 2006 *(131)*
Japan, women, mean age 57.9 years	115	Dietary record			47.2	Arai et al., 2000 as cited in Messina et al., 2006 *(131)*
Japan, women, 45–74 years old	34,195	FFQ			22.6	Ishihara et al., 2003 as cited in Messina et al., 2006 *(131)*
Japan, men, 45–74 years old	33,195	FFQ			23.5	
Japan, national survey, all ages	6,000	Dietary record			28.7	Nagata et al., 2000 as cited in Messina et al., 2006 *(131)*
Korea						
Korean population, ages not reported	Men: 3,224 Women: 3,475	Dietary interview (food eaten during 2 consecutive weekdays weighed and measured) IF levels obtained from studies published in peer-reviewed Korean journals	7.32	5.81	14.88[c]	Kim and Kwon 2001 *(132)*

Table 25 (continued)

Population		*n*	General Method of Estimate	Mean Intake, mg aglycone equivalents/day (median)			Reference
				Genistein	*Daidzein*	*Total Isoflavone*	
Korea	1–2 years	—	Daily food intake data from the 1998 National Nutrition Survey IF levels based on measurements of 220 Korean leguminous foods	5.2	8.0	14.5[d]	Surh et al., 2006 *(133)*
	3–6 years			3.0	5.2	8.9[d]	
	7–12 years			4.6	6.8	12.4[d]	
	13–19 years			4.4	5.0	10.1[d]	
	20–29 years			6.4	12.9	21.0[d]	
	30–49 years			8.3	22.1	33.6[d]	
	50–64 years			8.3	15.7	26.4[d]	
	≥65 years			7.0	10.3	18.8[d]	
China							
Chinese women, 37–61 years old		60	Dietary interview IF levels based on published literature	15.73 (17.92)[c]	14.9 (17.98)[c]	33.42 (39.26)[c]	Chen et al., 1999 *(134)*
China, women, mean age 49.9 years		45,694	FFQ			40.8	Yang et al., 2005 as cited in Messina et al., 2006 *(131)*
China, women, mean age 47.2 years		1,556	FFQ			40.9 (33.2)	Dai et al., 2001 as cited in Messina et al., 2006 *(131)*
China, women, 30–64 years old		1,823	FFQ			9.8 geometric mean	Frankenfeld et al., 2004 as cited in Messina et al., 2006 *(131)*
China, women, 20–65 years old		1,188	FFQ			17.7 (8.9)	Liu et al., 2004 as cited in Messina et al., 2006 *(131)*
China, women, mean age 48.0 years		652	FFQ			24.7	Zhang et al., 2004 as cited in Messina et al., 2006 *(131)*
China, men, 40–70+ years old		265	FFQ			75.7 (54.7)	Lee et al., 2003 as cited in Messina et al., 2006 *(131)*
Chinese living in Singapore, men and women, 45–74 years		Men: 14,776 Women: 71	FFQ IF levels measured in select soy foods[d]	15.90 mg/week **[2.27 mg/day]**[c]	15.65 mg/week **[2.24]**[c]	32.84 mg/week **[4.69 mg/day]**[c]	Seow et al., 1998 *(135)*

Table 25 (continued)

Population	*n*	*General Method of Estimate*	*Mean Intake, mg aglycone equivalents/day (median)*			*Reference*
			Genistein	*Daidzein*	*Total Isoflavone*	
Hong Kong						
Hong Kong, men, mean age 45.6 years	500	FFQ			14.5	Ho et al., 2000 as cited in Messina et al., 2006 *(131)*
Hong Kong, women, mean age 45.6 years	510	FFQ			11.0	
Hong Kong, women, mean age 37.5 years	293	FFQ			29.7	
Hong Kong, women, mean age 60.0 years	357	FFQ			21.9	
Hong Kong, women, 30–40 years old	132	FFQ			6.2	
Hong Kong, women, 50–61 years old	141	FFQ (n=141) and 23 dietary recalls during a 12-month period (n=133), IF levels based on analytical values in the Chinese University of Hong Kong Soy Isoflavone Database			7.8	Chan et al., 2007*(136)*

[a] Total isoflavone includes genistein, daidzein, formononetin, and biochanin A
[b] Total isoflavone intake was estimated by CERHR by adding the published genistein and daidzein estimated intakes present ranges; not that in the US, very little information is available to discern differences between men and women, but US NHANES data suggest.
[c] Total isoflavone includes genistein, daidzein, and glycitein
[d] Total isoflavone includes genistein, daidzein, glycitein, formononetin, and biochanin A
[e] Values provided in µmol/day and converted to mg/day by CERHR.

Gas chromatography/mass spectrometry (GC/MS) was used to measure isoflavones in 3-day composite 24-h urine samples. Intake of soy foods was correlated significantly with urinary genistein (r=0.40, *P*=0.0001) and the sum of isoflavonoids (r=0.39; *P*=0.0001). Intake of soy foods is significantly correlated with urinary genistein and the sum of isoflavonoids indicating that nearly all genistein exposure in humans occurs from ingestion of soy products *(57; 58)*.

Horn-Ross *et al.*, 2006 *(126)* studied a cohort of 195 members of the California Teachers Study who provided, over a 10 month period, four 24h dietary recalls, pre and post study FFQ and two 24h urine specimens. The author states that the pre- and post- study FFQ often referred to as reliability under the assumption that any reported changes in intake during this period are due to reporting errors rather than true changes in eating habits. Overall, validity correlations ranged from 0.41 to 0.55 between the FFQ and urinary isoflavone levels. Correlations between dietary intake and creatinine-adjusted urinary phytoestrogen levels to 1) 24h dietary recall- r = 0.41(genistein) and r=0.46, (daidzein) and 2) pre-study FFQ r=0.45, r=0.55. The study also addressed participants who did not use antibiotics within a year of the study (antibiotics can diminish the presence of gut microflora and potentially alter isoflavone metabolism) and found that correlations were improved (genistein r=0.74).

Jaceldo-Siegel *et al.*, 2008 *(137)* assessed soy protein intake estimates from FFQ in a sample of The Adventist Health Study-2 with a wide range of soy intakes. They evaluated the correlation coefficients between soy protein intake from 24h recalls and urinary isoflavonoids; these were 0.72 for daidzein, and 0.67 for genistein. Correlation coefficients of FFQ and urinary excretion were 0.50 for daidzein and 0.48 for genistein. The study authors suggest that in The Adventist Health Study-2 FFQ is a valid instrument for assessing soy protein in a population with a wide range of intakes.

Lee *et al.*, 2007 *(138)* assessed the validity and reproducibility of the FFQ used in the Shanghai Men's Health Study (SMHS) of dietary isoflavone intake. The correlation coefficient between the 2 FFQ administered 1 year apart was 0.5 for soy protein intake and ranged from 0.5 to 0.51 for isoflavone intake. The correlations of isoflavone intake from the second FFQ with those from the multiple 24-h dietary recall ranged from 0.38 (genistein) to 0.44 (glycitein). The author concludes that the SMHS FFQ can reliably and accurately measure intake of isoflavones.

The study by Nagata *et al.*, 2006 *(102)* described earlier included an assessment of the correlation between soy intake as determined by a 5-day diet record and isoflavone concentrations in cord serum and maternal urine. After controlling for covariates, maternal intake of dietary isoflavones was significantly correlated with cord serum isoflavone levels for both genistein (0.27, *P*=0.001) and daidzein (0.25, *P*=0.002). Dietary intake was moderately correlated with maternal urinary isoflavones during pregnancy. For example, at 29 weeks of gestation the correlations between intake and urinary isoflavones were 0.30 for genistein (*P*=0.0001) and 0.27 for daidzein (*P*=0.0004) at the 29th week after controlling for covariates.

1.2.2.8 Estimated Isoflavone Intake in Infants

While most estimates of adult exposure were based on dietary surveys, infant exposures were based on isoflavone levels measured in soy formula and assumed body weight and formula intake (**Table 26**). Depending on infant age, isoflavone equivalents intake for US infants was estimated at 1.9–11.5 mg/kg bw/day. The UK Ministry of Agriculture, Fisheries, and Food estimated isoflavone + conjugate intake in 1–2-month-old and 4–6-month-old infants based on survey results, average body weight, and intake data.

Table 26. Estimated Isoflavone Intakes in Infants Exclusively Fed Soy Formula

Age	Assumptions			Isoflavone Intake, aglycone equivalents					Reference
	Formula Intake, L/day	Body Weight, kg	Total Isoflavones, mg/L	mg/day	mg/kg bw/day[a]				
				Total	Total	Genistein	Daidzein	Glycitein	
US									
1 month	0.950[b]	4.8[b]	20.9–47	19.9–44.7	4.1–9.3	2.4–6.2	1.4–2.7	0.3–0.5	Table 9
3 months	0.980[b]	5.9[b]	20.9–47	20.5–46.1	3.5–7.8	2.0–5.2	1.2–2.2	0.3–0.4	
6 months	1.000[b]	7.4[b]	20.9–47	20.9–47.0	2.8–6.4	1.6–4.2	1.0–1.8	0.2–0.3	
12 months	1.000[b]	9.2[b]		20.9–47.0	2.3–5.1	1.3–3.4	0.8–1.5	0.2–0.3	
1 week	500–550	2.5–3.8	45[c]	22.5–24.8	5.7–7.3				Setchell et al., 1998 *(6)*
1 month	700–800	2.9–5.0	45[c]	31.5–36.0	6.0–11.9				
2 months	800–830	3.6–5.9	45[c]	36.0–37.0	6.1–10.0				
4 months	800–1000	4.8–7.5	45[c]	41.0–45.0	6.0–9.3				
Not specified			~25–30		5–12				Murphy et al., 1997 *(82)*
Not specified	1.0	4.5		30.5[d]	~7				Franke et al., 1998 *(81)*
UK									
1–2 months				28–34	5				Friar and Walker 1998 *(96)* as cited in Mortensen et al., 2009 *(14)*
4–6 months				28–34	4.5				
4–6 months	0.600				3.7				Hoey et al., 2004 *(87)*
Europe									
4–5 months (Switzerland)					3.4–13.5				Rupp et al., 2000 as cited in Mortensen 2009 *(14)*
> 5 months (Switzerland)					20 (maximum)				
New Zealand									
< 1 month		3		9.1	3.0				Irvine et al., 1998a, 1998b *(90; 91)*
1 month		4		14.1	3.8				
2 months		5		16.6	3.3				
4 months		7		20.0	2.9				

Table 26 (continued)

Age	Assumptions			Isoflavone Intake, aglycone equivalents					Reference
	Formula Intake, L/day	Body Weight, kg	Total Isoflavones, mg/L	mg/day	mg/kg bw/day[a]				
				Total	Total	Genistein	Daidzein	Glycitein	
South America									
0–2 weeks (Brazil)		~3		5.9–18.3	2.0–6.1				Genovese and Lajolo 2002 *(88)*
2–8 weeks (Brazil)		~4		6.6–26.2	1.7–6.6				
2–3 months (Brazil)		~5		8.2–26.2	1.6–5.2				
3–6 months (Brazil)		~6.5		9.2–34.9	1.4–5.4				

[a] Individual isoflavone intake values (mg/L) for infants at 1 month to 12 months of age: genistein (12.1 to ~31.2), daidzein (7.1 to ~13.5) and glycitein (1.7 to ~2.4).
[b] Assumptions on formula intake and body weight are based on the 2008 edition of the US EPA Child-Specific Exposure Factors Handbook *(139)*. Intake estimates of breastmilk are presented in the EPA handbook, but these estimates are similar to formula intakes at the 95% percentile (see Kersting et al., 1998 *(140)* as cited in *(92)*). Breastmilk intake estimates represent the upper percentile reported as mean plus 2 standard deviations. Body weights are the recommended mean values for girls and boys presented in the US EPA handbook.
[c] Based on a soy formula concentration of 45 µg/L. **[The unit of µg appears to be an error; units are listed as mg/L earlier in the report.]**

Isoflavone + conjugate intake was estimated at 5 mg aglycone equivalents/kg bw/day in 1–2-month-old infants and 4.5 mg aglycone equivalents/kg bw/day in 4–6-month-old infants. A comparison of estimated isoflavone equivalents intake from soy formulas available in different countries is included in **Table 26**, which also includes estimates of individual isoflavone equivalents levels based on mean levels detected in formula. **Table 27** presents a comparison of estimated total isoflavone intake in a 1-month old infant fed soy formula, casein-based formula, and breast milk.

Based on information presented above, mean or median genistein + conjugate exposures are estimated at ≤1 mg aglycone equivalents/day (0.01 mg aglycone equivalents/kg bw/day assuming a 70 kg bw) in US adults with no specified dietary preferences, 10–15 mg aglycone equivalents/day (0.1–0.2 mg aglycone equivalents/kg bw/day) in semi-vegetarian or vegetarians in 1 US survey, and 5–50 mg aglycone equivalents/day (0.1–1 mg aglycone equivalents/kg bw/day) in Asian adults. Based on mean mg/kg bw/day intake estimates, infants fed soy formula can be exposed to isoflavone + conjugate levels that are 2–3 orders of magnitude higher than US adults with low isoflavone exposure, 1–2 orders of magnitude higher than vegetarians in Western countries, and within the same order of magnitude to 1 order of magnitude higher than Asians. Exposure comparisons of isoflavone + conjugate intake in infants and adults based on food or formula intake were fairly consistent with conclusions based on blood isoflavone levels.

1.3 Utility of Data

As noted previously, the Expert Panel report is not intended to serve as a comprehensive inventory of every study that reports estimated daily isoflavone intake or levels in urine or blood. Instead, the focus is to summarize studies that are designed to be the most representative of typical intake or exposures, such as those that rely on NHANES data for US populations, or others based on large sample sizes. In addition, smaller studies are cited as they help inform isoflavone exposures in populations of special interest, such as Asian countries or vegetarian populations with relatively high usage of soy products.

There are numerous databases that present the isoflavone content of food items (reviewed in Schwarz *et al.*, 2009 *(59)*. The USDA maintains an extensive database that lists the isoflavone content of 557 food items, including several brands of soy formula and food items which may contain soy ingredients *(60)*. The isoflavone databases are the primary basis from which estimates of daily intakes in adults are derived. Studies summarizing daily intakes in adults and infants are presented in **Table 25**. For the US population, there are two recent studies that estimate isoflavone intake in adults who participated in the dietary recall portion of the NHANES and can be considered nationally representative estimates (Haytowitz *et al.*, 2009 *(66)* and Chun *et al.*, 2007 *(121)*). In addition, other U.S. studies have estimated intake in individuals, mostly women, enrolled in other research studies such as the Framingham Offspring Study (de Kleijn *et al.*, 2001 *(124)*), California Teachers Study cohort (Horn-Ross *et al.*, 2006 *(126)*), and the Family Risk Assessment Program (Tseng et a. 2008 *(68)*). A number of smaller studies have estimated isoflavone intake in special populations, including Hawaiian women (Maskarinec *et al.*, 1998 *(127)*) and participants in a case-control study of men with prostate cancer (Strom *et al.*, 1999 *(125)*). Kirk *et al.*, 1999 *(123)* compared isoflavone intake in a small sample of omnivorous, semi-vegetarian, and vegetarian men and women from a naturopathic university in Bellevue, Washington.

Table 27. Estimated Intake of Total Isoflavones in Infants at 1-Month of Age

Intake	*Formula Intake, L/day*	*Body Weight, kg*	*Total Isoflavones (as fed), mg/L*	*Total Isoflavone Intake, Aglycone Equivalents*		*Reference*
				mg/day	*mg/kg bw/day*	
Soy-based formula	0.950[a]	4.8[a]	20.9–47	19.9–44.7	4.1–9.3	**Table 26**
Casein-based formula	0.950	4.8[a]	0.001–0.08	0.001–0.076	0.0002–0.0158	**Table 11** Knight et al., 1998 *(89)*; Kuhnle et al., 2008 *(65)*
Breastfed (omniverous)	0.950[a]	4.8[a]	< LOD–0.0056	≤ 0.005	≤ 0.0011	**Table 11** Friar and Walker 1998 *(96)*; Setchell et al., 1998 *(6)*
Breastfed (vegetarian)	0.950[a]	4.8[a]	0.001–0.032	0.001–0.030	0.0002–0.0063	**Table 11** Friar and Walker 1998 *(96)*
Breastfed (after maternal soy challenge)	0.950[a]	4.8[a]	0.0013–0.52	0.001–0.494	0.0003–0.1029	**Table 11** Franke et al., 2006 *(95)*; Franke et al., 1998 *(81)*
Cow's milk[b]	0.950[a]	4.8[a]	0.005–0.350	0.005–0.333	0.0010–0.0693	**Table 11** King et al., 1997; Antignac et al., 2003 as cited in Mortensen et al., 2009 *(14)*

[a] Assumptions on formula and breastmilk intake, and body weight are based on the 2008 edition of the US EPA Child-Specific Exposure Factors Handbook *(139)*. Breastmilk intake estimates represent the upper percentile reported as mean plus 2 standard deviations. Intake estimates of breastmilk are presented in the EPA handbook and these estimates are similar to formula intakes at the 95% percentile (see Kersting et al., 1998 *(140)* as cited in *(92)*). Body weights are the recommended mean values for girls and boys presented in the US EPA handbook.

[b] Presented for comparison purposes only as cow's milk is not recommended for infants < 1 year of age.

A large number of studies have measured isoflavone levels in urine and blood. In July 2008, the CDC released the "National Report on Biochemical Indicators of Diet and Nutrition in the U.S. Population 1999-2002" *(115)* that included information on urinary concentrations of genistein, daidzein, and equol in ~5350 Americans age 6 years and older who took part in CDC's National Health and Nutrition Examination Survey (NHANES) during all or part of the four-year period from 1999 through 2002. The National Report does not include blood-based measurements of isoflavones, but in 2003, researchers from the CDC published serum levels of isoflavones in a non-representative subset of 209 adults who participated in NHANES *(107)*. Studies are also available that present blood or urine levels for Europeans, Japanese (including pregnant women), and vegetarians/vegans.

Exposures of infants to isoflavones and their conjugates through consumption of soy formulas have been estimated based on levels of isoflavones + conjugates measured in formulas, formula intakes, and infant body weights (**Table 26**). Estimated intakes of isoflavones have also been calculated for breastfed infants and those who consume a cow's milk-based formula (**Table 27**). Two US studies have compared blood levels of isoflavones in infants fed soy formulas to levels measured in infants fed cow milk or human milk (Cao *et al.*, 2009 *(94)* and Setchell *et al.*, 1997 *(84)*, summarized in **Table 15**).

The available data provide a good foundation for estimating approximate exposure and dose within broad populations or within individuals when the soy formula and the infants' weight and age are known.

1.4 Summary of Human Exposure Data

In children and adults, exposures to isoflavones occur through consumption of soy foods such as tofu, soy milk, soy flour, textured soy protein, tempeh, and miso *(25)*. Infants can be exposed by consuming soy-based infant formula, the breast milk of mothers who consume soy products, or by use of soy in weaning or "transition" foods. Soy oils or soy sauces contain little-to-no genistein *(15; 64)*. Soy protein can be used in baked goods, breakfast cereals, pasta, beverages, toppings, meat, poultry, fish products, and dairy-type products including imitation milk and cheese *(27; 28)*. Soybean derivatives are present in 60% of processed foods available from UK supermarkets *(3)*. The percentage of processed foods containing soybeans in the US is not known. Exposure to genistein can also occur through soy supplements marketed for the beneficial effects on health, such as improved cardiovascular health and treatment of menopausal symptoms *(9)*.

Based on sales of soy products, it appears that exposures to soy isoflavones in the US is increasing and will continue to increase. The Soyfoods Association of America reported soyfood sales have increased from $300 million to over $4 billion between 1992 and 2008, attributing this increase to new soy food categories being introduced, soy foods being repositioned in the market place, and new customers selecting soy for health and philosophical reasons *(27)*.

The primary isoflavones detected in soy products, including soybeans and soy formula, are derived from genistein, daidzein, and to a smaller extent, glycitein. These isoflavones are often referred to as phytoestrogens because of their ability to bind to estrogen receptors and display weak estrogenic activity compared to estradiol (at least based on *in vitro* model systems), with a relative estrogenic potency of genistein > daidzein > glycitein *(3; 5; 141)*.

The majority of isoflavones in soy formula and unfermented soy products are conjugated to sugar molecules to form the glycosides genistin, daidzin, and glycitin *(142; 143)*. Glucose groups in glycoside compounds can also be esterified with acetyl or malonyl groups to form acetyl- or malonyl glycosides *(3)* (**Figure 1**). The terms genistein, daidzein, and glycitein refer specifically to the unconjugated (aglycone) forms of the isoflavones. Small amounts of genistein and daidzein (3.2–5.8%) are present in soy products in their unconjugated (aglycone) forms *(13)*. As a result of bacterial hydrolysis during fermentation, aglycones represent a larger proportion of the isoflavones in fermented soy products such as miso, tempeh, and soybean paste *(3; 15)*. Because glycosidic compounds are rapidly deconjugated in the gut to form the biologically active aglycone compound, exposure to a particular isoflavone is theoretically the sum of the aglycone and respective glycoside compound concentrations converted on the basis of molecular weight *(3; 5)*. Unfortunately, there is an inconsistency in the literature where many studies do not clarify whether the presented isoflavone levels were normalized on an aglycone basis. Failure to convert the major glycosides, i.e., genistin, daidzin, glycitin, to the appropriate aglycone equivalents can overestimate isoflavone levels or intake by ~1.6-fold. Information in the Expert Panel report is normalized to aglycone equivalents where feasible.

Isoflavone levels in soybeans can vary as a result of crop strain, geographic location, climate, and growing conditions *(3; 6)*. Heating of soy products can cause decarboxylation, deacetylation, or deglycosylation of glycosides with decomposition of malonyl compounds to their respective acetylglycosides *(3; 6)*. Except for alcohol extraction, processing soybeans does not usually reduce isoflavone content *(15)*.

Soy formula refers to infant food made using soy protein isolate and other components such as corn syrup, vegetable oils, and sugar *(9)*. Decades ago soy formula included soy flour. However, in the 1950s and 1960s, cases of altered thyroid function, mostly goiter, were reported in infants fed soy formula. This problem was eliminated by adding more iodine to the formulas and replacing soy flour with soy protein isolate. Although the early reports of goiter in infants fed soy formula have mostly ceased since manufacturers began supplementing soy formula with iodine in 1959, there is still concern that use of soy formula in infants with congenital hypothyroidism may decrease the effectiveness of thyroid hormone replacement therapy, i.e., L-thyroxin. The soy protein isolate is fortified with L-methionine, L-carnitine, and taurine and other nutrients *(8; 42)*. Soy protein isolate includes phytates (1.5%), which bind minerals, and protease inhibitors, which have antitrypsin, antichymotrypsin, and antielastin properties *(42)*. Phosphorus, calcium, iron, and zinc are added to soy formula to compensate for phytate binding of minerals. Heat applied during the processing of soy protein removes 80–90% of protease inhibitor activity. Aluminum is present in soy formulas because of the addition of mineral salts.

Many aspects of infant formula use are unknown, including what percent of infants are exclusively fed formula compared to what percent are fed a mixture of infant formula and breast milk. It is also unknown what proportion of formula-fed infants are exclusively fed soy formula. A 1998 infant-feeding survey conducted by Ross Products Division indicated that 18% of infants are fed soy formula during the first year of life *(32)*. According to market data and hospital discharge records, another estimate was that 25% of newborns in the US are fed soy formula *(33)*. A study conducted at Yale University assessed formula changes in 189 breast-fed infants and 184 formula-fed infants and reported that 23% of infants in the study received soy formula sometime during the first 4 months of life *(34)*. Based on recent market data, sales of soy formula in the US represent ~12% of the US infant formula dollar

sales (personal communication with Robert Rankin, Manager of Regulatory and Technical Affairs at the IFC, October 13, 2009). In the US between 1999 and 2009, estimates of total soy infant formula fed decreased from 22.5% to 12.7% calculated based on total formula sold corrected for differences in formula cost. i.e., expressed in equivalent feeding units (public comment from the International Formula Council (IFC), received December 3, 2009 and personal communication with Dr. Haley Curtis Stevens, IFC). When sales are considered as a surrogate measure of actual reported usage, these data provide a lower indication of usage compared to other frequently cited estimates. The usage and sales of soy formula vary geographically ranging from 2 to 7% of infant formula sales in the UK, Italy, and France , 13% in New Zealand *(35; 36)*, to 31.5% in Israel *(37)*.

Commonly cited reasons for use of soy formula are to feed infants who are allergic to dairy products or are intolerant of lactose, galactose, or cow-milk protein *(38; 39)*. In May 2008, the American Academy of Pediatrics (AAP) released an updated policy statement on the use of soy protein-based formulas *(42)*. The overall conclusion of the AAP was that, although isolated soy protein-based formulas may be used to provide nutrition for normal growth and development in term infants, there are few indications for their use in place of cow milk-based formula. The only real indications for use are instances where the family prefers a vegetarian diet or for the management of infants with galactosemia or primary lactase deficiency (rare). Soy formula is not currently recommended for preterm infants. Similar conclusions were reached in 2006 by the European Society for Paediatric Gastroenterology Hepatology and Nutrition (ESPGHAN) Committee on Nutrition *(36)*.

A number of studies in the US and abroad have measured total isoflavone levels in infant formulas (**Table 9**). For formulas from the US, the range of total isoflavone levels reported in reconstituted or "ready-to-feed" formulas was 20.9–47 mg/L formula *(6; 81)*. When normalized to aglycone equivalents, genistein is the predominant isoflavone found in soy formula (~58-67%), followed by daidzein (~29-34%) and glycitein (~5-8%). In contrast to the isoflavone content of soy beans and other soy products such as soy supplements or soy protein isolates, the isoflavone content in soy formula is less variable. The range of total isoflavones content in soy formula samples collected in the US and other countries is 10-47 mg/L (**Table 10**) *(6; 88)*.

Isoflavone exposure through soy formula intake has been estimated in the US and other countries based on total isoflavone levels measured in soy formulas and assumptions of formula intakes and infant body weights. In the US, total isoflavone intake by infants was estimated at 2.3–9.3 mg/kg bw/day, depending on age of the infant (**Table 26**), the estimated intake for genistein, expressed in aglycone equivalents, ranges from 1.3 to 6.2 mg/kg bw/day. These intakes are several orders of magnitude greater than infants who consume breast milk or a cows milk-based formula (**Table 27**).

Soy formula fed infants have higher daily intakes of genistein and other isoflavones compared to other populations (excluding regular consumers of soy supplements) (**Table 28**). However, differences in strategies used to develop the intake estimates and sample representativeness limit the ability to compare across studies, especially for those based on dietary surveys. In addition, isoflavone intake seems to be inherently highly variable in adult populations and there is support for the notion that this variability cannot solely be attributed to differences in study methods. Recognizing these caveats, the relative ranking of total isoflavone intake appears to be infants exclusively fed soy formula > vegans > Japanese consuming a traditional diet > vegetarians > soy consumers.

Table 28. Comparison of Estimated Intake of Genistein and Total Isoflavones in Infants Fed Soy Formula to Other Populations

Population		Daily Intake, mg/kg bw/day*		Reference
		Genistein	Total Isoflavone	
US infants (soy formula)		1.3–6.2	2.3–9.3	Table 26
US adults (general)		0.005[a] – 0.056[b]	0.0097[a] – 0.096[b]	[a] Haytowitz et al., 2009 *(66)* b Tseng et al., 2008 *(68)*
US vegetarians		0.14	0.21	Kirk et al., 1999 *(123)*
European men	Not soy consumers	0.005	0.009	Mulligan et al., 2007 *(128)*
	Soy consumers	0.057	0.100	
European women	Not soy consumers	0.004	0.007	
	Soy consumers	0.062	0.112	
Vegans (UK)		—	1.07	Friar and Walker, 1988 *(96)* as cited in Mortensen et al., 2009 *(14)*
Japanese		0.077[a] – 0.43[b]	0.67[b]	[a] Fukutake et al., 1996 *(144)* as cited in Fitzpatrick, 1998 *(129)* [b] Arai et al., 2000 *(114)*

*Daily intakes for adults were based on mg/day estimates presented in Table 25 divided by 70 kg body weight.

Mean blood-based levels of isoflavones in infants fed soy formulas are considerably higher than other populations, including vegans and Japanese adults (Table 29). For example, concentrations of total genistein in whole blood samples from US infants fed soy formula are 1455 ng/ml at the 75th percentile *(94)*. This value is almost 5 times higher than the maximum genistein concentration detected in plasma in a small study of Japanese men, n=6 *(109)*. The mean level of genistein in the blood of the soy formula fed infants reported by Cao *et al.*, 2009 *(94)*, of 757 ng/ml is almost 20-times higher than the mean level of genistein detected in a sample of vegetarians and vegans in Oxford, England *(113)*. Average blood levels of total genistein in the soy formula-fed infants are ~160-times higher than the mean levels of total genistein in omnivorous adults in the US reported by Valentin-Blasini *et al.*, 2003 *(107)* (757 ng/ml versus 4.7 ng/ml). A similar pattern is observed based on urinary concentrations of isoflavones (Table 30).

Table 30. Comparison of Urinary levels of Genistein, Daidzein, and Equol in Infants Fed Soy Formula to the General US Population

Population	n	Urinary Concentration (geometric mean), µg/L or ng/ml			Reference
		Genistein	Daidzein	Equol	
US infants Fed soy formula	27	5891	5097	2.3	Cao et al., 2009 *(94)*
US children Ages 6–11 years	721–726	33.8	88.1	11.5	U.S. CDC, 2008 *(115)*
US adults Ages 40–59 years	951	23.7	52.3	8.39	U.S. CDC, 2008 *(115)*

Table 29. Comparison of Blood-Based Levels of Genistein and Daidzein in Infants Fed Soy Formula to Other Populations

Population	*n*	*Sample*	*Average Total Isoflavone Concentration, nM [ng/ml]*		*Reference*
			Genistein	*Daidzein*	
US infants (soy formula)	27	Whole blood (spots)	2801 **[757]**, geometric mean 3296 **[891]**, median 5384.5 **[1455]**, 75t h percentile	1007 **[256]**, geometric mean 1081 **[275]**, median 2040 **[519]**, 75t h percentile	Cao et al., 2009 *(94)*
	7	Plasma	2531 **[684]**	1160 **[295]**	Setchell et al., 1997 *(84)*
US infants(cow milk formula)	30	Whole blood (spots)	52.5 **[14.2]**, geometric mean	**[5.5]**, geometric mean	Cao et al., 2009 *(94)*
	7	Plasma	11.7 **[3.16]**, mean	8.1 **[2.06]**, mean	Setchell et al., 1997 *(84)*
US infants (breastfed)	20	Whole blood (spots)	40 **[10.8]**, geometric mean	20.8 **[5.3]**, geometric mean	Cao et al., 2009 *(94)*
	7	Plasma	10.3 **[2.77]**	5.9 **[1.49]**	Setchell et al., 1997 *(84)*
Omnivorous adults in US	209	Serum	17.4 **[4.7]** < LOD – **[203]**, range	15.3 **[3.9]** < LOD – **[162]**, range	Valentin-Blasini et al., 2003 *(107)*
Vegetarians, Finland	14	Plasma	17.1 **[4.6]**	18.5 **[4.7]**	Adlercreutz et al., 1994 *(109)*
Vegans & vegetarians (Oxford, UK)	70	Plasma	148 **[40]**	78.7 **[20]**	Peeters et al., 2007 *(113)*
Japanese women at delivery	51 a or 194 b	Serum	26.6 **[7.2]** a – 116.5 **[31.5]** b	7.1 **[1.8]** a – 50.2 **[12.8]** b	a Todaka et al., 2005 *(103)* b Nagata et al., 2006 *(102)*
Japanese cord serum at delivery	51 a or 194 b	Serum	71.8 **[19.4]** a – 126.9 **[34.3]** b	16.9 **[4.3]** a – 38.6 **[9.81]** b	
Japanese men (traditional diet)	6	Plasma	90.4 **[24]**-1204 **[325]**, range	58.3 **[15]**-924 **[235]**, range	Adlercreutz et al., 1994 *(109)*

2.0 PHARMACOKINETCS AND GENERAL TOXICOLOGY

2.1 Absorption, Distribution, Metabolism, and Elimination

This section describes the toxicokinetics and metabolism of the major isoflavones found in soy formula and other soy foods, genistein and daidzein. Toxicokinetic data are also presented for glycitein when possible although relatively few studies have evaluated this isoflavone. A large number publications discuss the administration, distribution, metabolism, and elimination (ADME), including the report "Phytoestrogens and Health" prepared by the UK Committee on Toxicity of Chemicals in Food, Consumer Products and the Environment *(3)* and reviews by Whitten and Patisaul, 2001 *(108)*, Cassidy *et al. (145)* Nielsen, 2007 *(143)*, Prasain and Barnes, 2007 *(146)*, and Larkin *et al.*, 2008 *(1)*.

Genistein, daidzein, and glycitein exist mainly in their glycosidic forms in unfermented soy foods. Before isoflavone glycosides can be absorbed into the systemic circulation, they must first be hydrolyzed to their aglycones, which have greater hydrophobicity and lower molecular weight. Prior to absorption, most genistein and daidzein are conjugated with glucuronic acid by uridine diphosphate (UDP)-glucuronosyltransferases; a smaller amount is conjugated to sulfate by sulfotransferases. Conjugation of isoflavones can also occur in liver. The glucuronide and sulfate conjugates enter the systemic circulation, and the majority of isoflavone compounds in the circulation are present in the conjugated form. Toxicokinetics and metabolism data in humans and experimental animals indicate that genistein and daidzein are absorbed into the systemic circulation of infants and adults.

Conjugated isoflavones undergo enterohepatic circulation, and on return to the intestine, they are deconjugated by bacteria with β-glucuronidase or arylsulfatase activity. The metabolites may be reabsorbed or further metabolized by gut microflora. Isoflavones can undergo further biotransformation that ultimately leads to the formation of 6′-hydroxy-*O*-desmethylangolensin from genistein and *O*-desmethylangolensin from daidzein. The metabolic profile varies among humans, with some individuals producing little or no *O*-desmethylangolensin or equol, an intermediate metabolite of daidzein that is biologically active.

The detection of genistein, daidzein, and equol in serum, urine, and breast milk in humans and experimental animals indicate that genistein and daidzein are absorbed into the systemic circulation of infants and adults. Isoflavones distribute to fetal fluids in humans and experimental animals and a limited number of studies in humans indicate that amniotic fluid or cord blood concentrations of genistein, daidzein, and equol are similar to concentrations in maternal blood. Details of the human and experimental animal studies on which these conclusions are based are presented in the sections below.

2.1.1 Humans

The detection of genistein, daidzein, and equol in serum, urine, amniotic fluid, cord blood, and breast milk in humans demonstrate fetal exposure and absorption into the systemic circulation of infants and adults. Relatively few studies include measurement of glycitein and it has not been measured in biomonitoring studies of the general population conducted by the CDC as part of NHANES *(115)* or measured in the plasma or urine of soy formula-fed infants *(84; 91; 94; 95)*. If glycitein is measured in humans, it is generally following intentional dosing of subjects with a soy protein or isoflavone supplement. There are few human studies in infants or children that present data relevant to toxicokinetics and metabolism Therefore all studies dealing with phamarcokinetics and metabolism in infants or children are described in this section. Review articles were referenced to summarize the most relevant studies in adults.

2.1.1.1 Absorption

Isoflavones exist mainly as glycosides in unfermented soy foods. Due to their high water solubility and molecular weight, isoflavone glycosides are not readily absorbed across the gastrointestinal tract (reviewed in *(1; 3; 147)*). For absorption to occur, isoflavones must first be hydrolyzed to their aglycones, which have some hydrophobicity and lower molecular weights. Aglycones undergo passive diffusion across the small and large intestinal brush border *(1)*. Glycosidase activity occurs in food product (by endogenous enzymes or those added during processing), in cells of the gastrointestinal mucosa, or colon microbes and isoflavones can be measured in blood within an hour of soy ingestion *(1; 148)*.

Mammalian β-glucosidases identified in the small intestine include broad spectrum cytosolic β-glucosidase enzyme and the membrane-bound lactase phlorizin hydrolase (LPH) enzyme. LPH can be found on the luminal side of the brush border of the small intestine and cause the release of aglycones which diffuse into the epithelial cells. As noted in the review by Larkin *et al.*, 2008 *(1)*, hydrolysis within the intestine would require initial uptake of the glycoside form which has been demonstrated for other flavonoids, but not conclusively for isoflavones *(149)*.

The toxicokinetics and bioavailability of isoflavones, particularly for genistein and daidzein, are relatively well-studied in adults. Cassidy *et al.*, 2006 *(145)* reviewed 16 human studies[8] that measured isoflavone bioavailability in plasma, urine, or feces and used all of the data points from these studies to calculate summary estimates of C_{max}, t_{max}, $t_{1/2}$, AUC, urinary excretion, and fecal excretion. Each of the 16 studies summarized by Cassidy *et al.*, 2006 *(145)* did not necessarily include values for all of these parameters, i.e., some focused on urinary or fecal excretion and did not assess C_{max}, t_{max}, or $t_{1/2}$. This set of studies was also considered in a subsequent review article by Nielsen and Williamson, 2007 *(143)*, although there were some differences between the overall goals of the review articles that resulted in slight variations in the published summary values (personal communication with Gary Williamson, October 16, 2009). In brief, the Nielsen and Williamson, 2007 *(143)* review was most interested in comparing the data for genistein and daidzein to genistin and daidzin and presented summary values derived from studies in the same concentration range. This resulted in slight differences in the published summary values, i.e., normalized C_{max} for genistein=0.64 μM in Nielsen and Williamson, 2007 *(143)* compared to 0.49 μM in Cassidy *et al.*, 2006 *(145)*. In addition, there are small differences in the percent urinary excretion (average value and range) between the two review articles, i.e., 20% in Nielsen and Williamson, 2007 *(143)* compared to 19% in Cassidy *et al.*, 2006 *(145)*.

The studies considered in the reviews by Cassidy *et al.*, 2006 *(145)* and Nielsen and Williamson, 2007 *(143)* included men and women, both pre- and post-menopausal.

Based on the summary of these studies presented in Nielsen and Williamson, 2007 *(143)* and Cassidy *et al.*, 2006 *(145)*, pharmacokinetic parameters (C_{max}, t_{max}, $t_{1/2}$, AUC) are similar for genistein and daidzein. For genistein, the average t_{max} was 5.7 hours (range of 3.5−9.3 hours) and for daidzein, it was 6.2 hours (range of 4.0−8.3 hours). These estimates are consistent with the range of peak values of ~6−8 hours reported by others for genistein and daidzein following ingestion of soy or isoflavones

[8] The sixteen studies summarized were: King and Bursill, 1998 [150], Richelle *et al.*, 2002 [151], Zubik and Meydani, 2003 [152], Busby *et al.*, 2002 [153], Setchell *et al.*, 2001 [76], Setchell *et al.*, 2003 [154], Xu *et al.*, 1994 [155], Xu *et al.*, 1995 [156], Zhang *et al.*, 1999 [157], Zheng *et al.*, 2003 [158], Rowland *et al.*, 2003 [159], Faughnan *et al.*, 2004 [160], Tew *et al.*, 1996 [161], Watanabe *et al.*, 1998 [162], and Shelnutt *et al.*, 2002 [163].

(*(112)*; reviewed in *(1; 3; 108)*). The maximum concentrations (C_{max}) of genistein and daidzein, when normalized to ingestion of 1 µmol/kg body weight, were also similar at 0.49 *(145)* or 0.64 µM *(143)* or 0.50 *(143)* or 0.54 µM *(145)*, respectively. As discussed above, there were slight variations in the published summary values between the two reviews.

The average half-life of elimination ($t_{1/2}$) estimates from reviews by Nielsen and Williamson 2007 *(143)* and Cassidy *et al.*, 2006 *(145)* did not differ appreciably between genistein, 9.5 hours (range 6.1-17 hours), and daidzein, 7.7 hours (range 4.2–16 hours). These estimates are generally similar to those presented in a recent review by Larkin *et al.*, 2008 *(1)*; 3–9 hours for daidzein and 8–11 hours for genistein after intake of soy foods or pure isoflavone glycosides.

The AUC values, when normalized to ingestion of 1 µmol/kg body weight, were 11 µmol*hour/L for genistein and 18 µmol*hour/L for daidzein, although Cassidy *et al.*, 2006 *(145)* noted these values as having a high degree of uncertainty because of considerable variability between studies. **[The basis for the variability noted in Cassidy et al., 2006 is not specifically described, but plausible explanations are variability in factors such as the isoflavone composition, administered dose, food matrix, sample collection time, and the number of time-points evaluated. For example, if measurements are not made at or near t_{max}, then under-estimates of AUC are possible. Or, if too few time points are assessed, then AUC can be over-estimated.]** Absorption half-lives for both daidzin and genistin, presumably as glucuronides and sulfates of daidzein and genistein, were reported at ~1–3 hours following intake of foods containing ≤210 mg of each isoflavone or providing doses of ≤2 mg/kg bw/day of each isoflavone (*(164; 165)*; reviewed in *(108)*).

In 2001, Whitten and Patisaul *(108)* published a review of phytoestrogens that included a summary of human plasma concentrations following a single dietary dose of genistein or daidzein. Single soy meals providing genistein and daidzein doses of 0.07–1.3 mg/kg and 0.06–1.2 mg/kg produced peak plasma genistein and daidzein concentrations of 0.100–2.15 µM and 0.06–2.22 µM, respectively. Plasma C_{max} concentrations of genistein and daidzein are reached within 4–8 hour, with an absorption half-life of 1–3 hour (**Table 31**). The excretion half-life ranges from 3 to 8 hour. Based on a variety of approaches, daidzein bioavailability (16–66%) appeared to greater than genistein bioavailability (5–37%). Data, albeit limited, from children is consistent with these values. Irvine *et al.* (1998) presented data from four soy formula-fed infants in whom urinary recovery of daidzein and genistein was reported to be 38 ± 4% and 13 ± 3% of daily isoflavone intake, respectively.

Setchell *et al.*, 2001 *(76)* reported that < 4% of total genistein in plasma is circulating in the unconjugated or "free" form in women. The percent unconjugated genistein in plasma was similar following administration of genistein (1.6–3.7%) or genistin (1.1–1.5%). For plasma daidzein, a somewhat higher percent was present in the unconjugated form following administration of daidzein (2.7–8.4%) compared to administration of daidzin (1.1–1.7%). Rufer *et al.*, 2008 *(166)* reported a higher percentage of total daidzein present in the unconjugated form based on a study in men, although the percentages did not vary based on whether the aglycone or glucoside was administered. They reported 3.4–12.9% in the unconjugated form following ingestion of daidzein as the aglycone and 3.1–11% unconjugated following ingestion of daidzein in glucoside form. The fraction of total genistein and daidzein in circulation present in the unconjugated biologically active form is low, ranging from ~ 1–3% at steady state *(76)*, although the percentage as unconjugated maybe greater near C_{max} *(46)*.

Table 31. Estimates of Isoflavone Bioavailability in Human Subjects

Source	Dose [a], mg/kg/day	c_{max}, μM [µg/L]	t_{max}, hours	Absorption $t_{1/2}$, hours	Excretion $t_{1/2}$, hours	Bioavailability [b]	Reference
Genistein							
Soy milk	0.3–0.8					5–11%	Xu et al., 1994 *(155)* as cited in Whitten and Patisaul, 2001 *(108)*
	0.3–0.8					10–37%	Xu et al., 1995 *(156)* as cited in Whitten and Patisaul, 2001 *(108)*
	1.7			1.9→1.4	3.8	15%	Lu et al., 1995 *(165)* as cited in Whitten and Patisaul, 2001 *(108)*
Kinako	0.4	2.4 **[649]**	6		8.4	20%	Watanabe et al., 1998 *(162)* as cited in Whitten and Patisaul, 2001 *(108)*
Tofu	0.6					13–16%	Tew et al., 1996 *(161)* as cited in Whitten and Patisaul, 2001 *(108)*
Soy flour	1.0	4.1 **[1108]**	8.4		4.7	22%	King et al., 1998 *(150)* as cited in Whitten and Patisaul, 2001 *(108)*
Daidzein							
Soy milk	0.4–1.2					16–32%	Xu et al., 1995 *(156)* as cited in Whitten and Patisaul, 2001 *(108)*
	0.4–1.2					20–24%	Xu et al., 1994 *(155)* as cited in Whitten and Patisaul, 2001 *(108)*
	1.7			1.5→2.5 (before and after one month)	2.9	47%	Lu et al., 1995 *(165)* as cited in Whitten and Patisaul, 2001 *(108)*
Tofu	0.4					49%	Tew et al.,1996 *(161)* as cited in Whitten and Patisaul, 2001 *(108)*
Kinako	0.4	1.6 **[407]**	6		5.8	56%	Watanabe et al., 1998 *(162)* as cited in Whitten and Patisaul, 2001 *(108)*
Soy flour	0.8	3.1 **[788]**	7.4		5.7	62%	King et al., 1998 *(150)* as cited in Whitten and Patisaul, 2001 *(108)*

[a] Dose gives the estimated intake in mg/kg/day.
[b] Estimates of isoflavone bioavailability following ingestion of soy-based product.
Modified from Table 9 and Table 10 in Whitten and Patisaul, 2001 *(108)*.

In some reviews, such as the one conducted by Whitten and Patisaul *(108)*, recoveries were considered to represent bioavailability. Note, estimation of bioavailability in this way is reasonable if urine collections cover 5 half-lives after a single dose, or a "dosing interval" for continuous dosing. Because recovery of daidzein and its metabolites usually exceeds that of genistein and its metabolites, it has been concluded that daidzein is generally more bioavailable than genistein; however, this conclusion is not supported by blood data that sometimes indicate higher levels of total genistein than total daidzein.

Huang *et al.*, 2008 *(167)* used *in vitro* and *in vivo* models to evaluate the topical delivery of isoflavones for clinical applications. Genistein and daidzein were moderately absorbed by the skin and that skin absorption was generally higher for genistein compared to daidzein.

Bioavailability based on administered form

Studies in humans that report the bioavailability and other pharmacokinetic parameters of isoflavones ingested as glycosides versus aglycones have reported conflicting findings (reviewed in *(3; 143; 145)*). Attempts to reconcile the conflicting findings are complicated by differences in experimental approaches used in these studies, i.e., administered form of the isoflavone, dosage, etc., as well as the different definitions of bioavailability used in the isoflavone literature. Prasain and Barnes *(146)* discussed the various approaches used to assess the bioavailability of isoflavones. In brief, bioavailability is frequently evaluated differently in the pharmaceutical and nutritional literatures. In pharmacology (and toxicology) the term absolute bioavailability refers to the ratio of AUC after oral ingestion to the AUC after systemic administration, i.e., IV injection. This is the fraction of the compound absorbed through non-intravenous administration compared with the corresponding intravenous administration of the same drug. This approach may underestimate bioavailability at the tissue level if circulating isoflavones in the conjugated form are converted to aglycones by β-glucuronidases and sulfatases secreted by cells within target tissues.

In the nutrition literature, bioavailability is often assessed by the percent of administered isoflavone that is recovered in the urine. Higher degrees of recovery in urine are interpreted as more bioavailability based on the assumption that the isoflavone must have been present in blood prior to reaching the kidney and ultimately being excreted in urine. Prasain and Barnes, 2007 *(146)* noted a number of limitations in the use of urine to assess isoflavone bioavailability. First, compounds with short half-lives do not reside in the body long. In such cases, bioavailability could be limited even if a high degree of recovery in the urine was observed. In contrast, compounds that are retained in tissues may be excreted more slowly or to a lesser extent in urine and therefore considered less bioavailable. Also, compounds that undergo extensive reabsorption in the proximal tubules of the kidney may be interpreted as having poor bioavailability because they may not appear in high levels in the urine. Prasain and Barnes, 2007 cite the example of bile acids as a case where 95-98% of the oral dose is taken up from the intestines yet there is little appearance in the urine due to extensive reabsorption in the kidney.

Bloedon *et al.*, 2002 *(168)* conducted a pharmacokinetic study of two formulations of purified isoflavones in postmenopausal women and calculated relative bioavailability of the formulations using total genistein measurements from plasma AUC and for urinary excretion. The formulations were administered to deliver the same dose levels of genistein or daidzein, but differed in composition based on percentage of isoflavones present in unconjugated form and constitution of genistein, daidzein, and glycitein. Formulation A contained 100% unconjugated isoflavones (87% genistein, 12% daidzein, and 1%

glycitein), and formulation "B" contained 70% unconjugated isoflavones (44% genistein, 23% daidzein, and 2% glycitein). Relative bioavailability for plasma and urine was calculated at the ratio of formulation A/B. In general, the relative bioavailabilities were similar using the two approaches although it appeared that for genistein relative bioavailability based on urine was lower compared to plasma AUC and for daidzein, relative bioavailability based on urinary excretion was higher than estimates based on plasma AUC. For example, at administered doses of genistein of 4, 8, and 16 mg/kg the relative bioavailabilities based on plasma AUC of total genistein were 1.12, 1.11, and 1.28, respectively. The relative bioavailability estimates for these administered doses based on urinary excretion of total genistein were 1.01, 1.06, and 0.938. For administered doses of daidzein of ~1 and 2 mg/kg, the relative bioavailabilities based on plasma AUC of total daidzein were ~0.9 and ~1.0, respectively. The relative bioavailability estimates for these administered doses based on urinary excretion of total daidzein were 0.76 and 0.71.

Based on their review of 16 human studies, Nielsen and Williamson, 2007 *(143)* concluded that despite the apparently contradictory findings in the literature, the data are consistent enough to conclude that (1) at equivalent doses, the C_{max} is higher for genistein and daidzein following administration as glucosides compared to aglycones, and (2) the half-life is not significantly different for aglycone and glucoside. In addition, they concluded that deglycosylation is required for absorption but does not appear to be a rate-limiting step. Studies by Setchell *et al.*, 2001 *(76)* and Rufer *et al.*, 2008 *(166)* suggest the percent of genistein and daidzein circulating in the unconjugated forms do not differ based on administered form. These conclusions support the assumption used by others that because glycosides are deconjugated in the gut to form the active aglycones, exposure to a particular isoflavone (e.g., genistein) is theoretically the sum of the aglycone and respective glycoside compound concentrations converted on the basis of molecular weight *(3; 5; 6; 17)*.

While there is no clear explanation for the conflicting findings on bioavailability of the aglycone and glucoside one factor may be the differences in the type of isoflavone preparations administered to subjects, e.g, soybean extracts containing mixtures of isoflavones, purified single isoflavone, ingestion in tablet or liquid form *(142)*. In addition, comparing the conclusions on bioavailability across studies is difficult because of the variety of indices used to assess bioavailability, e.g., relative AUC, c_{max}, t_{max}, urinary excretion and recovery, etc. **[The Expert Panel notes that bioavailability in pharmacology and toxicology refers to the ratio of AUC after oral ingestion to the AUC after iv injection. The term bioavailability is often used in different ways by different authors.]** Several of the more frequently cited studies in discussions of bioavailability based on the administered form are described below.

Setchell *et al.*, 2001 *(76)* reported a longer time to achieve maximal plasma concentrations (t_{max}) of genistein and daidzein following ingestion of 50 mg of these isoflavones as glucosides (genistin=9.3h; genistein=5.2h; daidzin=9.0h; daidzein=6.6h), presumably due to the time required for hydrolysis of the glucose moiety from genistin and daidzin prior to absorption. The C_{max} and relative AUC normalized for dose for daidzein was higher following administration of the glucoside compared to the aglycone (AUC=4.52 ± 0.49 μg/(ml·h) versus 2.94 ± 0.22 μg/(ml·h). In addition, the half-life of elimination for daidzein was approximately twice as long following administration of the aglycone (9.3 hours versus 4.59 hours). In contrast, there appeared to be little difference following ingestion of genistin or genistein for plasma genistein for C_{max} (341 ng/ml and 341 ng/ml) relative AUC (4.95 μg/(ml·h) verus 4.54 μg/(ml·h)) and or half-life for genistein (7.0 hours versus 6.78 hours). A study conducted by Rufer *et al.*, 2008 *(166)* also provides support for increased bioavailability of daidzein following administration of

the glucoside based on a higher AUC and C_{max} compared to administration of the aglycone. **[Rufer et al., 2008 *(166)* did not assess genistein.]** Following administration of 1 mg/kg bw of daidzin or daidzein, the respective AUC values were 38.5 µmol·h/L and 8.3 µmol·h/L and the respective C_{max} values were 2.54 ng/ml and 0.43 ng/ml. However, in contrast to Setchell *et al.*, 2001 *(76)*, Rufer *et al.*, 2008 *(166)* did not observe any difference in t_{max} leading the authors to suggest that the hydrolysis of the *O*-glucosidic bond is not a rate-limiting factor in absorption. Proposed factors to explain a greater bioavailability of the glucoside include a protective effect of the glycoside moiety to prevent degradation from intestinal bacteria and a greater water solubility of the glucoside compared to the aglycone *(143; 166)*.

In contrast, Izumi *et al.*, 2000 *(169)* reported that genistein and daidzein ingested as aglycones were absorbed more quickly and in higher amounts than when ingested as glucosides. Subjects ingested single, low- and high-dose tablets of isoflavones as aglycones or glucosides. Other subjects ingested aglycone or glucoside tablets for 4-weeks. Intakes of genistein and daidzein were similar, but not identical, between the aglycone and glucoside treatment groups. For example, the genistein and daidzein intakes in the single, low dose isoflavone aglycone groups were 0.78 and 0.92 mmol while in the single, low dose glucoside single groups the intakes for genistein and daidzein were 0.9 and 0.8 mmol. Depending on the specific isoflavone and administered dose, the maximum plasma concentrations were ~3 to 7 times greater following ingestion of isoflavone aglycones compared to ingestion of isoflavone glucosides. Izumi *et al.*, 2000 *(169)* also reported that plasma concentrations of both genistein and daidzein were higher at the mid-point and end of the 4-week period when the tablets (taken daily) were administered as isoflavones aglycones compared to glucosides. Hutchins *et al.*, 1995 *(119)* concluded that the bioavailability of isoflavones may be increased when ingested as aglycones based on finding significantly higher recoveries of genistein and daidzein in urine collected from men following ingestion of fermented soy product with a higher isoflavone aglycone content (tempeh) compared to an unfermented soy product (soybean) for a 9-day period. The percent recoveries of genistein and daidzein in urine from men on the tempeh diet was 1.9% and 9.7%, respectively, compared to 1.3% and 5.7% from men on the soybean diet.

Yet other studies have reported no differences in t_{max}, C_{max}, AUC, or half-life when isoflavones are ingested as glycosides or aglycones *(151; 152)*. **[In Zubik et al., 2003 *(152)*, C_{max} and AUC (but not t_{max}), were significantly higher for daidzein following administration of the aglycone. The authors note that daidzein intake was higher in the aglycone treatment group (0.0624 nmol total intake) compared to the glucoside treatment group (0.0492 nmol total intake).]** With respect to conclusions based on urinary measurements, Xu *et al.*, 2000 *(16)* observed that bioavailability of isoflavones, assessed by urinary reovery as a percentage of intake did not vary when they were administered to women as foods containing a high percentage of aglycones (e.g., tempeh) compared to a high percentage of glycosides (e.g., soybeans). **[The authors commented that the percentage of ingested dose of daidzein and genistein recovered in urine appeared smaller, "lesser bioavailability" for tempeh (9% and 38% for genistein and daidzein) compared to other soy products such as tofu, cooked soybean, or texturized vegetable protein (13-16% and 45-51% for genistein and daidzein) but this difference was not statistically significant. The authors noted that detecting a significant difference would have been difficult given the small sample size used and interindividual variation].** Maskarinec *et al.*, 2008 *(170)* reported no difference in bioavailability estimated from overnight urinary isoflavonoid excretion following ingestion of one serving of fermented soy food (miso soup) or unfermented soy food (soy milk) with equivalent isoflavone content in a group of 21 women of Japanese ancestry. The authors

conclude that these preliminary results do not support the hypothesis that consumption of fermented soy foods leads to greater isoflavone exposure than ingestion of unfermented soy foods.

Impact of food matrix

The impact of food matrix is another factor that has been considered in assessing the impact of administered form on isoflavone bioavailability; however, the literature is not consistent. One conclusion presented in a review by Cassidy *et al.*, 2006 *(142)* that considered this issue is that the amount of isoflavone, and hence, bioavailability can be more influenced by storage and processing factors rather than food matrix used to deliver the isoflavones, e.g., cooked soy beans, tofu, soy milk powder, etc. Cassidy *et al.*, 2006 *(171)* conducted a detailed evaluation of genistein and daidzein kinetics after ingestion of different soy foods. This evaluation is summarized in **Table 32**. The rate and extent of isoflavone absorption is greater from liquid than from solid matrices *(171; 172)*.

Toxicokinetics of unconjugated and total genistein and daidzein

Most of the available pharmacokinetic information for humans is based on measurement of the total isoflavone (unconjugated + conjugated). However, two studies conducted by the same research group, briefly described below, have compared the pharmacokinetics of unconjugated and total genistein, daidzein, and glycitein following administration of isoflavones in post-menopausal women *(168)* or men *(153)* (**Table 33** and **Table 34**). The reported terminal "pseudo" half-lives for total genistein (in women an average of 10.1 hours; range of 7.8-13.4; in men an average of 9.2 hours, range of 6.1-12.6) and total daidzein (in women an average of 10.8 hours; range of 5.7-16.1; in men an average of 8.2 hours, range of 4.2-16) were similar to the average values presented in Nielsen and Williamson 2007 *(143)* and Cassidy *et al.*, 2006 *(145)* based on reviews of 16 human studies (genistein=average of 9.5 hours, range of 6.1-17; daidzein=average of 7.7 hours, range of 4.2-16).

Correlations of isoflavone concentrations between plasma, urine, or other fluid matrices

The gold standard for an estimate of systemic exposure following a given dose of the compound(s) of interest is the AUC. The primary value of correlation studies should be to determine the strength of the relationship between surrogate measures of exposure, such as C_{max} and urinary recovery (preferablybased on the total mass of aglycone and metabolites eliminated in a collection interval covering five half-lives and expressed relative to the administered dose). In this regard, the study by Bloedon *et al.* (2002) *(168)* in post-menopausal women and Busby *et al.*, 2002 *(153)* in healthy men are most helful.

Both studies utilized the same study design and formulations. Two different isoflavone preparations were used in these studies. Formulation A contained 100% unconjugated isoflavones (87% genistein, 12% daidzein, and 1% glycitein), whereas Formulation B contained 70% unconjugated isoflavones (44% genistein, 23% daidzein, and 2% glycitein). Four doses of genistein were used (2, 4, 8, and 16 mg/kg body wt) for each of two isoflavone formulations. The authors stated that these genistein doses range from 2 to >20-times the amount of total isoflavone present in the average Japanese diet. The amounts of daidzein and glycitein that were provided by formulation A at each genistein dose were 0.28, 0.55, 1.1, and 2.2 mg/kg bw for daidzein and 0.014, 0.029, 0.057, and 0.11 mg/kg bw for glycitein. For formulation B, the doses of daidzein were 1.0, 2.1, 4.2, and 6.8 mg/kg bw and for glycitein they were 0.08, 0.17, 0.34, and 0.68 mg/kg bw. The total number of dose groups was eight, four for Formulation A and four for Formulation B. Plasma samples were obtained at the following time points: 0 (immediately before administration of the formulation), 0.5, 1, 1.5, 3, 4.5, 6, 9, 12, 15, 18, and 24 hours post-dose.

Table 32. Total Genistein and Daidzein Kinetics After Ingestion of Soy Foods

Food		*C_{max}, µM/mg dose* [mg/L/mg dose]	*t_{max}, hour*	*Half-life, hour*	*AUC, µM-hour/ mg dose* mg-hour/L/mg dose]	*Vd/F, L/kg bw*	*Cl/F, L/hour*
Daidzein							
Soy Milk	Women	2.19 ± 0.72 **[0.56 ± 0.18]**	6.1 ± 1.7	8.0 ± 1.2	22.09 ± 4.29 **[5.61 ± 1.09]**	1.53 ± 0.57	8.47 ± 2.54
	Men	1.79 ± 0.63 **[0.45 ± 0.16]**	6.5 ± 2.2	7.5 ± 1.4	22.08 ± 9.25 **[5.61 ± 2.35]**	1.65 ± 0.71	11.49 ± 5.32
Bread rolls	Women	1.09 ± 0.39 **[0.28 ± 0.10]**	8.4 ± 1.3	9.4 ± 2.8	15.28 ± 3.76 **[3.88 ± 0.96]**	2.07 ± 0.84	9.86 ± 2.08
	Men	1.21 ± 0.35 **[0.31 ± 0.09]**	8.0 ± 1.6	8.3 ± 2.1	16.29 ± 4.65 **[4.14 ± 1.18]**	1.66 ± 0.58	10.97 ± 3.32
Tempeh burger	Women	2.33 ± 1.23 **[0.59 ± 0.31]**	8.4 ± 0.8	7.8 ± 1.3	27.13 ± 8.99 **[6.89 ± 2.28]**	1.33 ± 0.61	6.55 ± 1.69
	Men	1.32 ± 0.33 **[0.34 ± 0.08]**	8.0 ± 2.0	7.3 ± 1.4	19.79 ± 7.87 **[5.03 ± 2.00]**	1.73 ± 0.80	14.87 ± 9.36
Tukey-Kramer *P*	Men v. women	0.01	NS	NS	NS	NS	0.0001
	Milk v. rolls	<0.0001	<0.0005	0.001	<0.0001	0.002	NS
	Milk v. burger	NS	0.0005	NS	NS	NS	NS
	Rolls v. burger	<0.0001	NS	0.0004	<0.0001	0.002	NS
Genistein							
Soy Milk	Women	4.07 ± 1.89 **[1.10 ± 0.51]**	5.6 ± 1.7	9.9 ± 2.2	50.01 ± 21.31 **[13.50 ± 5.75]**	0.72 ± 0.37	3.31 ± 1.73
	Men	3.47 ± 1.95 **[0.94 ± 0.53]**	6.6 ± 2.1	9.8 ± 1.6	51.26 ± 37.00 **[13.84 ± 9.99]**	1.04 ± 0.67	5.24 ± 2.78
Bread rolls	Women	1.36 ± 0.56 **[0.37 ± 0.15]**	7.8 ± 1,0	10.9 ± 2.9	18.35 ± 6.42 **[4.95 ± 1.73]**	1.90 ± 0.86	7.81 ± 3.26
	Men	1.38 ± 0.59 **[0.37 ± 0.16]**	7.2 ± 2.1	10.5 ± 2.1	22.98 ± 14.12 **[6.20 ± 3.81]**	1.83 ± 1.07	7.90 ± 3.76
Tempeh burger	Women	2.35 ± 1.03 **[0.63 ± 0.28]**	7.2 ± 1.3	9.4 ± 2.1	32.28 ± 14.26 **[8.72 ± 3.85]**	1.12 ± 0.46	6.58 ± 3.07
	Men	1.59 ± 0.58 **[0.43 ± 0.16]**	7.5 ± 1.8	9.6 ± 1.4	26.91 ± 13.50 **[7.27 ± 3.65]**	1.55 ± 0.66	8.73 ± 4.05
Tukey-Kramer *P*	Men v. women	NS	NS	NS	NS	NS	NS
	Milk v. rolls	<0.0001	0.0001	NS	<0.0001	<0.0005	<0.0005
	Milk v. burger	<0.0001	NS	NS	<0.0001	NS	<0.0005
	Rolls v. burger	0.008	NS	NS	0.02	0.02	NS

Data presented as mean ± SD, n = 21/sex.
NS = not signficiant.
From Cassidy et al., 2006 *(171)*.

Table 33. Genistein: Pharmacokinetic Comparison of Unconjugated ("Free") and Conjugated ("Total")

Sample and Dosing Information	*Dose, mg/kg bw*	*Form*	t_{max}, *hours*	C_{max}, *nM*	k_{el}	$t_{1/2}$, *hours*	V_d, *L/kg bw*	Cl_p, *L/kg bw-hour*	*AUC, nmol·h/L*
Healthy postmenopausal women (3/group) with low soy product intake ingested a formulation containing 100% unconjugated isoflavones (87% genistein). Bloedon et al., 2002 *(168)*	2	free	3.3 ± 2.0	47 ± 19	0.35 ± 0.06	2.0 ± 0.30	145 ± 113	47.7 ± 34.0	182 ± 116
	4		3.5 ± 2.4	99 ± 79	0.21 ± 0.13	4.2 ± 2.46	153 ± 95	24.5 ± 1.7	544 ± 106
	8		8.3 ± 6.4	117 ± 36	0.13 ± 0.03	5.7 ± 1.34	318 ± 292	36.9 ± 17.8	1028 ± 621
	16		2.5 ± 1.7	204 ± 39	0.30 ± 0.18	3.2 ± 2.30	205 ± 119	47.6 ± 29.4	1326 ± 505
	Average (range)		**4.4 (2.5–8.3)**			**3.8 (2.0–5.7)**			
	2	total	4.5 ± 1.5	3440 ± 1425	0.11 ± 0.02	6.5 ± 1.1	1.91 ± 0.76	0.208 ± 0.097	35,394 ± 15,174
	4		7.5 ± 5.4	8545 ± 621	0.07 ± 0.04	12.4 ± 7.9	1.23 ± 0.08	0.085 ± 0.038	129,072 ± 23,261
	8		9.5 ± 4.3	14,172 ± 4492	0.07 ± 0.04	13.4 ± 7.7	1.71 ± 0.68	0.108 ± 0.073	212,952 ± 102,646
	16		6.5 ± 2.3	28,158 ± 15,954	0.08 ± 0.03	10.0 ± 3.8	1.69 ± 0.64	0.147 ± 0.117	432,978 ± 254,319
	Average (range)		**7.0 (4.5–9.5)**			**10.6 (6.5–13.4)**			
Healthy postmenopausal women (3/group) with low soy product intake ingested a formulation containing 70% unconjugated isoflavones (44% genistein). Bloedon et al., 2002 *(168)*	2	free	2.3 ± 1.9	126 ± 95	0.45 ± 0.16	1.7 ± 0.5	71.3 ± 59.7	26.6 ± 15.1	327 ± 162
	4		2.5 ± 3.0	155 ± 109	0.23	3.8	66	14.4	806 ± 616
	8		1.0 ± 0.5	134 ± 29	0.11 ± 0.04	7.3 ± 3.2	441 ± 397	36.9 ± 29.9	695 ± 371
	16		1.0 ± 0.5	360 ± 221	0.36 ± 0.17	2.2 ± 0.8	130 ± 91	45.9 ± 23.5	2229 ± 2252
	Average (range)		**1.7 (1.0–2.5)**			**3.7 (1.7–7.3)**			
	2	total	3.00 ± 1.50	5638 ± 2369	0.070 ± 0.019	10.5 ± 3.3	1.49 ± 0.61	0.107 ± 0.058	64,651 ± 29,448
	4		3.50 ± 2.29	8672 ± 1869	0.073 ± 0.026	10.6 ± 4.7	1.57 ± 0.39	0.119 ± 0.063	115,572 ± 48,857
	8		4.50 ± 1.50	15,235 ± 1665	0.092 ± 0.019	7.76 ± 1.55	1.53 ± 0.43	0.135 ± 0.012	192,600 ± 25,404
	16		4.52 ± 1.52	25,413 ± 8733	0.079 ± 0.009	8.91 ± 1.08	2.12 ± 0.86	0.171 ± 0.082	337,949 ± 172,529
	Average (range)		**4.1 (2.5-6.5)**			**9.1 (5.7-12.3)**			

Table 33 (continued)

Sample and Dosing Information	Dose, mg/kg bw	Form	t_{max}, hours	C_{max}, nM	k_{el}	$t_{1/2}$, hours	V_d, L/kg bw	Cl_p, L/kg bw-hour	AUC, nmol·h/L
Healthy men (3/group) abstained form eating soy products and ingested a formulation containing ≥97% unconjugated isoflavones (90% genistein). Busby et al., 2002 *(153)*	1, 2, 4	free							
	8		6.5 ± 3.8	131 ± 21	0.428	1.9	104	38.5	
	16		2.8 ± 2.8	66 ± 31	0.333	2.3	877	258	
	Average (range)		**4.7 (2.8–6.5)**			**2.1 (1.9–2.3)**			
	1	total	5.5 ± 0.9	929 ± 88	0.091 ± 0.034	8.2 ± 2.5	3.6 ± 0.4	0.326 ± 0.088	
	2		7.5 ± 1.5	2095 ± 451	0.073 ± 0.025	10.3 ± 3.8	3.7 ± 1.3	0.253 ± 0.016	
	4		6.5 ± 3.8	4418 ± 2502	0.103 ± 0.030	7.2 ± 2.5	3.5 ± 2.9	0.381 ± 0.364	
	8		8.0 ± 2.3	8037 ± 2203	0.076 ± 0.019	9.5 ± 2.1	2.9 ± 0.7	0.220 ± 0.075	
	16		4.7 ± 2.8	7594 ± 1384	0.085 ± 0.023	8.6 ± 2.5	6.4 ± 1.4	0.534 ± 0.152	
	Average (range)		**6.4 (4.7–8.0)**			**8.8 (7.2–10.3)**			
Healthy men (3/group) abstained from eating soy products and ingested [a] formulation containing ≥70% unconjugated isoflavones (43% genistein). Busby et al., 2002 *(153)*	1	free	6.0	74	0.443	1.6	15.9	7.0	
	2		5.0 ± 3.1	69 ± 33	0.209 ± 0.103	4.1 ± 2.5	112 ± 50	20.7 ± 9.6	
	4		2.7 ± 0.6	84 ± 14	0.141 ± 0.053	5.4 ± 1.8	186 ± 64	25.8 ± 10.2	
	8		3.5 ± 3.5	258 ± 134	0.295 ± 0.011	2.4 ± 0.1	99.0 ± 44.8	29.2 ± 13.5	
	16		2.5 ± 1.7	363 ± 213	0.317 ± 0.239	3.1 ± 1.9	226 ± 211	49.4 ± 43.8	
	Average (range)		**3.9 (2.5–6.0)**			**3.3 (1.6–5.4)**			
	1	total	5.7 ± 3.2	2729 ± 1710	0.076 ± 0.025	9.9 ± 3.3	2.2 ± 1.9	0.148 ± 0.093	
	2		3.7 ± 2.1	5492 ± 1516	0.083 ± 0.020	8.7 ± 2.4	1.7 ± 1.4	0.125 ± 0.066	
	4		6.0 ± 0.0	9479 ± 2053	0.116 ± 0.019	6.1 ± 1.0	1.1 ± 0.2	0.128 ± 0.046	
	8		4.5 ± 2.6	17,870 ± 2426	0.056 ± 0.009	12.6 ± 1.8	1.8 ± 0.3	0.100 ± 0.018	
	16		3.5 ± 1.7	27,460 ± 15,380	0.067 ± 0.012	10.6 ± 2.2	3.2 ± 2.6	0.218 ± 0.194	
	Average (range)		**4.7 (3.5–6.0)**			**9.6 (6.1–12.6)**			

$t_{1/2}$ = half-life; Cmax = maximum plasma concentration; t_{max} = time to cmax; k_{el} = terminal elimination rate constant; V_d = volume of distribution; Clp = apparent systemic clearance; AUC = area under the time-concentration curve.

Values presented as mean ± SD; values without a SD could be measured in fewer than 3 subjects. AUC values calculated by assigning a value of 0 to values below the limit of quantification. To convert nM to µg/L, multiply by 0.27.

Table 34. Daidzein: Pharmacokinetic Comparison of Unconjugated ("Free") and Conjugated ("Total")

Sample and Dosing Information	*Dose, mg/kg bw*	*Form*	*t_{max} hours*	*C_{max}, nM*	*k_{el}*	*$t_{1/2}$, hours*	*V_d, L/kg bw*	*Cl_p, L/kg bw-hour*	*AUC, nmol·h/L*
Healthy postmenopausal women (3/group) with low soy product intake ingested a formulation containing 100% unconjugated isoflavones (12% daidzein) Bloedon et al., 2002 *(168)*	0.55	Free							
	1.1		8.3 ± 6.4	22 ± 6	0.075 ± 0.056	21.2 ± 25.2	190 ± 93	10.8 ± 5.9	175 ± 114
	2.2		2.5 ± 1.7	35 ± 7	0.167 ± 0.083	4.7 ± 1.8	208 ± 54	33.9 ± 14.8	212 ± 96
	average (range)		**5.4 (2.5–8.3)**			**13.0 (4.7–21.2)**			
	0.55	Total	7.5 ± 5.4	1719 ± 1132	0.050 ± 0.020	16.1 ± 8.0	1.94 ± 1.04	0.084 ± 0.013	14104 ± 5972
	1.1		11.0 ± 1.7	1801 ± 419	0.090 ± 0.040	8.8 ± 4.0	1.74 ± 1.02	0.136 ± 0.038	25696 ± 9435
	2.2		5.5 ± 3.1	3114 ± 1579	0.069 ± 0.038	14.3 ± 11.5	2.31 ± 0.73	0.174 ± 0.127	46353 ± 30448
	average (range)		**8.0 (5.5–11.0)**			**13.1 (8.8–16.1)**			
Healthy postmenopausal women (3/group) with low soy product intake ingested a formulation containing 70% unconjugated isoflavones (23% daidzein). Bloedon et al., 2002 *(168)*	1.0	Free	2.5 ± 1.7	69 ± 34	0.160 ± 0.020	4.4 ± 0.5	81 ± 16	12.8 ± 1.4	269 ± 29
	2.1		1.5 ± 0.0	74 ± 38	0.173 ± 0.067	4.6 ± 2.1	128 ± 71	22.3 ± 14.9	451 ± 334
	4.2		1.2 ± 0.3	100 ± 38	0.108 ± 0.011	6.5 ± 0.7	147 ± 18	15.9 ± 3.6	679 ± 292
	8.4		2.3 ± 1.9	176 ± 105	0.194 ± 0.126	4.9 ± 3.4	245 ± 299	28.9 ± 19.2	1707 ± 1615
	average (range)		**1.9 (1.2–2.5)**			**5.1 (4.4–6.5)**			
	1.0	Total	2.5 ± 1.7	2521 ± 849	0.090 ± 0.032	8.3 ± 2.7	1.41 ± 0.63	0.116 ± 0.022	27602 ± 9457
	2.1		3.0 ± 1.5	3600 ± 482	0.099 ± 0.081	10.0 ± 5.5	1.76 ± 0.81	0.139 ± 0.049	47691 ± 11984
	4.2		4.5 ± 1.5	8973 ± 2950	0.058 ± 0.014	12.3 ± 2.7	3.28 ± 0.69	0.195 ± 0.075	134780 ± 47000
	8.4		6.5 ± 4.8	6488 ± 785	0.128 ± 0.032	5.7 ± 1.7	1.46 ± 0.33	0.182 ± 0.036	84602 ± 13009
	average (range)		**4.1 (2.5–6.5)**			**9.1 (5.7–12.3)**			
Healthy men (3/group) abstained from eating soy products and ingested a formulation containing ≥97% unconjugated isoflavones (90% genistein). Busby et al., 2002 *(153)*	0.44	Total	7.5 ± 2.6	0.583 ± 0.279	0.109	6.6	1.7	0.188	
	0.89		8.0 ± 2.6	0.906 ± 0.228	0.111 ± 0.075	8.1 ± 4.4	2.9 ± 1.4	0.252 ± 0.059	
	1.8		6.0 ± 1.5	1.315 ± 0.057	0.158 ± 0.020	4.4 ± 0.5	3.0 ± 0.02	0.471 ± 0.027	
	average (range)		**7.2 (6.0–8.0)**			**6.4 (4.4–8.1)**			

Table 34 (continued)

Sample and Dosing Information	*Dose, mg/kg bw*	*Form*	*t_{max} hours*	*C_{max}, nM*	*k_{el}*	*$t_{1/2}$, hours*	*V_d, L/kg bw*	*Cl_p, L/kg bw-hour*	*AUC, nmol·h/L*
Healthy men (3/group) abstained from eating soy products and ingested a formulation containing ≥70% unconjugated isoflavones (21% daidzein). Busby et al., 2002 *(153)*	0.49	Free	7.5	0.060[a]	0.328[a]	2.1[a]	15.0[a]	4.9[a]	
	0.98		3.2 ± 2.5	0.040 ± 0.010	0.13[a]	7.3[a]	93.4[a]	10.3[a]	
	2.0		3.5 ± 0.9	0.070 ± 0.012	0.266 ± 0.173	3.4 ± 1.9	72.4 ± 17.0	17.3 ± 6.7	
	3.9		2.5 ± 1.8	0.139 ± 0.027	0.167 ± 0.038	4.3 ± 1.1	111 ± 48	18.2 ± 8.9	
	7.8		2.7 ± 1.6	0.315 ± 0.261	0.306 ± 0.200	3.5 ± 2.9	134 ± 145	22.2 ± 11.8	
	average (range)		**3.9 (2.5–7.5)**			**3.7 (3.4–4.3)**			
	0.49	Total	6.0 ± 2.6	1.429 ± 0.767	0.123 ± 0.007	5.6 ± 0.3	1.3 ± 0.7	0.164 ± 0.082	
	0.98		4.2 ± 2.9	2.221 ± 0.640	0.171 ± 0.044	4.2 ± 0.9	1.0 ± 0.6	0.167 ± 0.064	
	2.0		6.0 ± 0.0	4.857 ± 0.384	0.139 ± 0.044	5.4 ± 2.1	1.2 ± 0.5	0.144 ± 0.015	
	3.9		4.0 ± 3.1	7.678 ± 1.961	0.051 ± 0.021	16.0 ± 8.6	2.7 ± 2.1	0.109 ± 0.038	
	7.8		5.0 ± 0.9	16.94 ± 8.711	0.118 ± 0.046	6.7 ± 3.4	1.4 ± 0.6	0.159 ± 0.088	
	average (range)		**5.0 (4.0–6.0)**			**7.6 (4.2–16.0)**			

C_{max} = maximum plasma concentration; t_{max} = time to C_{max}; k_{el} = terminal elimination rate constant; V_d = volume of distribution; Cl_p = apparent systemic clearance; AUC = area under the time-concentration curve. Values presented as mean ± SD; values without a SD could be measured in fewer than 3 subjects. AUC values calculated by assigning a value of 0 to values below the limit of quantification. To convert nM to µg/L, multiply by 0.27.

[a] Sample size of 1 or 2

Urine was collected over 24 hours post-dose at the following time points: immediately before administration of the formulation and at intervals ending 3, 6, 12, and 24 h post-dose. Plasma and urine samples were analyzed for free genistein, free daidzein, free glycitein, and their respective totals (free plus sulfate and glucuronide conjugates) fractions by HPLC.

Both studies demonstrated excellent correlation between dose administered and both C_{max} and AUC. In pre-menopausal women *(168)*, mean C_{max} values for total genistein and total daidzein increased linearly with increasing doses ($r^2 > 0.92$) for both formulations tested, which differed only in the daidzein content. The linearity of mean C_{max} values with increasing doses could be established for free daidzein in formulation B (~4-fold higher daidzein content relative to formulation A; $r^2=0.97$) and for free genistein in formulation A; ($r^2=0.96$). For AUC, mean AUC values increased linearly with increasing doses for total genistein and total daidzein from both formulations ($r^2 > 0.98$) and for free genistein and free daidzein as well ($r^2> 0.88$). Based on the 24-hour urinary recovery of genistein glucuronide and sulfate conjugates, the reported C_{max} and AUC values appear to reflect 10-14% of the administered dose. Although the absolute amount of genistein conjugates recovered in the urine appeared to be dose-dependent (urinary recovery as a percentage of dose was similar at each dose level), no correlations between urinary recovery and either C_{max} or AUC were reported. Similar results were observed in men *(153)*.

Weaker relationships were observed when urinary concentrations or recoveries were correlated with single blood or plasma concentrations rather than AUC values. Valentín-Blasini *et al.*, 2003 *(107)* measured serum levels of genistein, daidzein, equol, and *O*-desmethylangolensin from 209 samples taken from a nonrepresentative sample of adults who participated in NHANES III, 1988-1994. Serum levels were several times lower than the corresponding urine measurements and highly significant correlations between levels in urine and serum samples from the same persons were observed for genistein (r=0.79, *P*=0.0001), daidzein (r=0.72, *P*=0.0001), and *O*-desmethylangolensin (r=0.41, *P*=0.0007).

Setchel *et al.*, 2003 *(173)* reported statistically significant correlations of between genistein serum C_{max} and urinary excretion expressed as either concentration ($r^2=0.286$) or total output during a 24-hour period ($r^2=0.424$).These same correlations were lower and not statistically significant for daidzein, $r^2=0.085$ and 0.056 *(173)*. The authors concluded that because the data were considerably scattered, urinary isoflavone concentrations for a given individual provide only a crude estimate of intake and are limited as predictors of systemic bioavailability.

Mathey *et al.*, 2006 *(174)* measured isoflavones in the plasma and urine of post-menopausal women who ingested high quanities of soy isoflavones in a Prevastein extract for 30 or 60 days. The daily intake from the Prevastein was 100 mg of total isoflavones expressed as aglycone equivalents (~1.4 to 1.6 mg/kg bw/day) and was 55-75% genistein and 20–40% daidzein. The correlation coefficients between plasma and urine were generally low and ranged from 0.56 to 0.81 for genistein and 0.15 to 0.23 for daidzein, depending on whether the samples were collected at day 15 or day 30 of treatment. When all samples collected between 15 to 60 days of treatment were considered, the correlations were higher for both genistein (r=0.59) and daidzein (r=0.36).

Franke *et al.*, 2008 *(175)* reported that the correlation between plasma isoflavone and urinary isoflavone excretion rate (UIER) were significantly improved when time-based measurements covering the same time interval are used (i.e., AUC) rather than estimates that do not consider the time interval (i.e.,

plasma concentration and UIER). This conclusion was based on a study where Franke *et al.*, 2008 *(175)* used urinary excretion rate to assess how oral antibiotic use affects isoflavone bioavailability in chidren and adults. This study included data on the correlation between plasma and urine isoflavone values. The data related to antibiotic use in children and adults is discussed further in **Section 2.1.1.3** (see "Urinary exretion rate to estimate apparent bioavailability in infants, children, and adults") Eight men and six women consumed 10 g of soy nuts (~ 22.1 mg isoflavone aglycone equivalents). Blood samples were collected at 3-6 hours and 4-9 hours after soy intake. Each participant emptied their bladder shortly after the first blood draw and the urine sample for analysis was collected between the two blood draws. The authors did not adjust final urinary excretion rates for body weight because the body weights of study participants were similar. The correlation coefficient between isoflavone levels in urine and plasma was calculated using linear regression. Isoflavone levels in the two matrices were better correlated, r=0.93 (P<0.001) when AUC for both plasma and urine (both time-based units) were used rather than UIER (a time-based measure, nmol/h) and plasma isoflavone levels at first collection (a non-time-based unit, nM). The correlation between UIER and plasma level at first collection was 0.68 and was not statistically significant. Overall, the authors concluded that urine can be used as a reliable surrogate to estimate isoflavone intake and systemic exposure.

Nagata *et al.*, 2006 *(102)* evaluated isoflavone exposure in 194 Japanese women by estimating the dietary intake and measuring genistein, daidzein and equol in maternal urine **[creatinine normalized]**, serum during gestation and at delivery, as well as serum in umbilical cord samples. Isoflavones were measured using HPLC-MS/MS (for serum) or HPLC (urine) after the addition of β-glucuronidase/suflate to the samples. The geometric mean and range of concentrations of genistein, daidzein, and equol for maternal and cord blood serum samples are presented in Chapter 1 in **Table 9**. After controlling for covariates, genistein and daidzein in umbilical cord serum were highly correlated with levels in both maternal urine (r=0.63 **[r^2=0.40]**, P<0.0001 and r=0.58 **[r^2=0.34]**, P <0.0001 for genistein and daidzein, respectively) and serum (r=0.70 **[r^2=0.49]**, P<0.0001 and r=0.68 **[r^2=0.46]**, P<0.0001 for genistein and daidzein, respectively) at delivery.

Adlercreutz *et al.*, 1999 *(97)* measured genistein, daidzein, *O*-desmethylangolensin, and equol in maternal plasma, cord plasma, and amniotic fluid samples collected from seven Japanese women at delivery. Average levels of isoflavones and metabolites in cord blood and amniotic fluid were similar to maternal cord blood levels (see **Table 9** in Chapter 1). However, maternal plasma levels of genistein and daidzein were not significantly correlated with levels in either cord plasma ($r_{\text{maternal plasma·cord plasma}}$=0.338 for genistein **[$r^2$=0.11]**; 0.439 for daidzein **[r^2=0.19]**) or amniotic fluid ($r_{\text{maternal plasma·amniotic fluid}}$=0.530 for genistein **[$r^2$=0.28]**; 0.418 for daidzein **[r^2=0.17]**). Levels of *O*-desmethylangolensin and equol in maternal plasma correlated significantly with levels in cord plasma and amniotic fluid with correlations ranging from 0.967 to 0.998. Significant correlations for all the isoflavones and metabolites ranging from 0.889 to 0.998 were found between cord plasma and amniotic fluid. The authors concluded that phytoestrogens cross the placenta. Levels in fetuses were similar to maternal levels, and conjugation patterns were reportedly similar to those observed in Japanese individuals.

Two infant studies are relevant (described further below), but compared to the adult studies described above, correlations were determined between spot urine concentrations and spot blood/plasma levels. Cao *et al.*, 2009 *(94)* calculated correlations between genistein and daidzein levels in the urine **[spot, per unit volume]**, saliva, and blood of infants fed soy formula. The study design did not permit an

estimation of isoflavone dose, and the time of blood collection relative to feeding was not specified. Overall, coefficients of determination, r^2, between urine and saliva or urine and blood were less than 0.1. Thus, although statistically significant correlations between blood and spot urine concentrations were observed in this study, a spot urine concentration provided no information regarding the total systemic isoflavone exposure.

Franke *et al.*, 2006 *(95)* reported correlations between fluid levels in individual infants and mothers as well as between mothers and infants as part of a study designed to compare isoflavones patterns in the urine **[spot, creatinine normalized]** and plasma of breastfed infants and their mothers following maternal consumption of a soy protein beverage; three tofu-fed infants were included in the study for comparison. Isoflavone concentrations were significantly correlated within type of biological matrix for an individual and within mother-infant pair for breastfed infants: mother's milk versus mother's urine, r=0.661; mother's milk versus infant urine, r=0.775; mother's urine versus infant urine, r=0.863, and infant plasma versus infant urine, r=0.975. Thus, a relationship between blood/plasma isoflavone concentration and urinary concentrations exists in children as in adults.

Relationship between administered dose and blood and urinary isoflavone pharmacokinetic parameters

Cassidy *et al.*, 2006 *(145)* reviewed 16 human studies and concluded that C_{max} was the pharmacokinetic value that correlated the most with administered dose. Between doses of 0.2 and 59 µmol/kg body weight, the correlation with peak plasma concentration of genistein was r^2=0.974. For daidzein, the r^2 was 0.958 between 1 and 31 µmol/kg body weight. Weaker correlations of 0.730 and 0.716 were calculated for genistein and daidzein were administered as glucosides. No significant correlations were observed between administered dose and area under the curve, urinary excretion, or fecal excretion. A more detailed description of these 16 human studies and additional summary values are presented in Nielsen and Williamson *(143)*. Correlation coefficients were calculated between plasma C_{max} and administered doses of < ~8 µmol/kg bw of genistein (r^2=0.50) or daidzein (r^2=0.87) and administered doses of < ~6 µmol/kg bw genistin (r^2=0.73) or daidzin (r^2=0.72). **[These values were presented in the caption for Figure 3 in the Nielsen and Williamson review. The figure legends and figure caption do not match. CERHR assumed that the caption text stating "genistein, r^2=0.73; and daidzein, r^2=0.72" actually refers to correlation graphs presented for genistin and daidzin.]**

With respect to infants, assuming that the infants in the study by Franke *et al.*, 2006 *(95)* consumed approximately equivalent volumes of milk, the data from that study imply that a significant relationship also exists between isoflavone dose and concentration in blood and urine in this age group.

Many studies have measured genistein and daidzein in plasma, urine, bile or feces, saliva, breast milk, and amniotic fluid. A less extensive literature exists on the measurement of these isoflavones in other human fluids or tissues, and these studies are generally limited to assessment of the breast or prostate (reviewed in Larkin *et al.*, 2008 *(1)*). Setchell *et al.*, 2001, 2003 *(76; 173)* have reported relatively large volumes of distribution for genistein, daidzein, and glycitein indicating widespread distribution to tissues. Bloedon *et al.*, 2002 *(168)*and Busby *et al.*, 2002 *(153)* reported much higher volumes of distribution for unconjugated, or "free", genistein and daidzein compared to total genistein and daidzein (**Table 33** and **Table 34**) indicating tissue-level exposure to the biologically active forms.

Distribution to the embryo or fetus

In humans, genistein, daidzein, and equol can be measured in cord blood and amniotic fluid, indicating distribution to the embryo or fetus. Studies reporting the isoflavones concentrations in fetal and/or maternal compartments are summarized in **Table 13** and **Table 14** in Chapter 1. Other studies described in detail in Chapter 1 indicate that genistein, daidzein, and equol also distribute to breast milk and that breast milk concentrations increase following ingestion of soy foods (see **Table 17**). **[CERHR did not identify any studies that attempted to measure glycitein in breast milk, amniotic fluid, cord blood, or maternal blood collected at delivery.]**

Tissue distribution

Studies in humans reported that daidzein concentrations in prostate fluid were ~2–4 times higher than plasma levels and equol concentrations in prostate fluid were 44 times higher than in plasma (reviewed by *(108)*. Recent studies generally support this conclusion.

There do not appear to be any data concerning isoflavone concentrations in tissues or matrices beyond blood/plasma and urine in children.

Volume of distribution

A study by Setchell *et al.*, 2001 *(76)* of three healthy premenopausal women reported the mean volume of distribution normalized to bioavailability fraction (V_d/F), another measure of systemic exposure, for genistein and daidzein following single ingestion of 50 mg **[~0.8 mg/kg assuming a 60 kg bw]** of the compounds as aglycones and glycosides Following intake of each compound in its glycosidic form, the mean volume of distribution for the bioavailable fraction was reported at 161.1 L **[~2.7 L/kg bw assuming a 60 kg bw]** for genistein and 236.4 L **[~3.9 L/kg bw]** for daidzein. Ingestion of the glycosidic compounds resulted in volumes of distribution for the bioavailable fraction of 112.3 L **[~1.9 L/kg bw]** for genistein and 77 L **[~1.3 L/kg bw]** for daidzein. The V_d/F was large for both daidzein and genistein, indicating extensive tissue distribution. The V_d/F for daidzein was much higher than for genistein (236 L versus 161 L), which the authors noted provides an explanation of why genistein levels in plasma exceed daidzein concentrations when equivalent amounts of the two isoflavones are ingested. **[It does not appear that the authors conducted statistical analyses to determine whether the difference in V_d/F between genistein and daidzein were statistically significant.]** The V_d/F for glycitein was relatively high at 415 L.

Setchell *et al.*, 2003 *(173)* calculated the mean V_d/F in premenopausal women who ingested a single 0.4 or 0.8 mg/kg bw dose of [13C]genistein or [13C]daidzein on four separate occasions. Consistent with the earlier finding of Setchell *et al.*, 2001 *(76)*, *V_d/F was higher for daidzein compared to genistein although the differences were not statistically significant.* The average of the two visits when women ingested the low dose of 0.4 mg/kg bw was 224.1 L **[~3.7 L/kg bw assuming a 60 kg bw]** for genistein and 305.7 L **[~5.1 L/kg bw]** for daidzein. At the higher dose of 0.8 mg/kg bw, the average V_d was 243.1 L **[~4.1 L/kg bw assuming a 60 kg bw]** for genistein and 399.2 L **[~6.7 L/kg bw]** for daidzein. Differences between the 0.4 and 0.8 mg/kg bw dose groups were not statistically significant.

Bloedon *et al.*, 2002 *(168)* and Busby *et al.*, 2002 *(153)* reported that mean volumes of distribution for total genistein and daidzein were similar in men and women volunteers who consumed soy products containing 70–100% unconjugated isoflavones.**[Because the doses were administered orally, V_d**

is V_d/F.] However, they did not observe a larger V_d/F for daidzein compared to genistein like that reported by Setchell *et al.*, 2001 *(76)* and Setchell *et al.*, 2003 *(173)*. Detailed descriptions of these studies are provided in **Section 2.1.1** and results are summarized in **Table 33** and **Table 34**. Bloedon *et al.*, 2002*(168)* calculated V_d/F in postmenopausal women following administration of one of four formulations that provided different doses of unconjugated genistein (1 to 16 mg/kg bw) and daidzein (0.28 to 8.4 mg/kg bw). The range of average V_d/F for each group were 1.23 to 2.12 L/kg bw for total genistein and 1.41 to 3.28 for total daidzein. In the same studies, volumes of distribution were presented for unconjugated genistein and daidzein and these were higer. For the unconjugated isoflavones, the mean volumes of distribution were 66 to 441 L/kg for free genistein **[the low value is based on two subjects]** and 81.3–245 L/kg bw/day for free daidzein. Busby *et al.*, 2002 *(153)* calculated V_d/F in healthy men following administration of one of four formulations were used that provided different doses of unconjugated genistein (1 to 16 mg/kg bw) and daidzein (0.28 to 8.4 mg/kg bw). The range of average V_d/F for each group were 1.1 to 6.4 L/kg bw for total genistein and 1.0 to 3.38 for total daidzein. In the same studies, volumes of distribution were presented for unconjugated genistein and daidzein and these were higher. For the unconjugated isoflavones, the mean volumes of distribution were 15.9 to 877 L/kg bw for free genistein **[these values were based on either one or two subjects]** and 15–245 L/kg bw for free daidzein **[the "15" value is based on n=1]**. The study authors noted that the higher volumes of distribution for the free versus total isoflavones suggest that free genistein and daidzein are more likely to enter or be sequestered within tissues. **[There are problems in the reporting of volumes of distribution for the aglycones in these studies. The Expert Panel finds ranges of 16–877 and 15–245 L/kg bw/day to be implausible in this variability. In addition, volumes of distribution in the range of <10 L/kg bw in some reports are not consistent with volumes of distribution in the hundreds of L/kg bw in other reports. It is possible that the lower values were obtained from a mixture of aglycones and glucuronides with glucuronides predominating. The higher number may not take into consideration the bias toward the conjugated compounds in equilibrium.]**

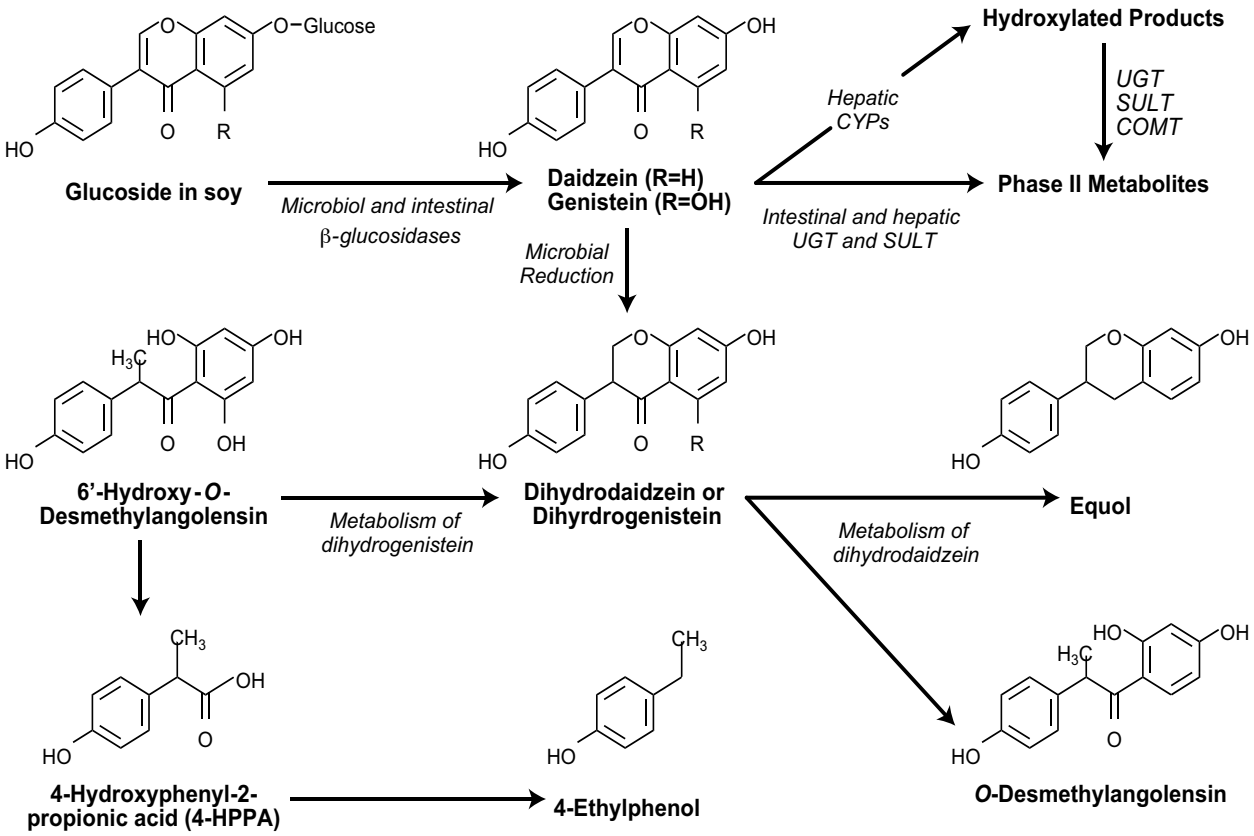
2.1.1.2 Metabolism

The metabolic fate of genistein, daidzein, and glycitein have not been completely characterized, but the major known metabolic pathways for genistein and daidzein are presented in **Figure 3**. In both animals and humans the major pathways are glucuronidation and sulfation.

Gu *et al.*, 2006 *(176)* compared the isoflavone profiles for genistein, daidzein, equol, and glycitein and other isoflavone metabolites in women, following ingestion of soy protein isolate (results are summarized in **Table 59**). In addition to equol, other metabolites measured were *O*-DMA, dihydrogenistein, and dihydrodaidzein. Among the ten women, urinary glucuronide conjugates constituted 85%, sulfate conjugates were 13.3% and the free aglycone was 0.1%.

Much less information is available on the metabolism of glycitein. Rufer *et al.*, 2007 *(177)* characterized the phase 1 metabolism of glycitein in rat liver microsomes, human liver microsomes, and human fecal flora. In rat liver microsomes, glycitein was converted to 10 metabolites with 8-hydroxy(OH)-GLY as the main metabolite. In human liver microsomes, glycitein was metabolized to six metabolites with 8-OH-GLY and 6-OH-daidzein being the major products. Following incubation with human fecal flora, glycitein was metabolized to four metabolites with 6-OH-daidzein as the main product. In addition, *in vivo* metabolism was studied in Sprague-Dawley rats. Three oxidative, 2 bacterial metabolites, and 6-OH-daidzein were identified in rats treated with a single dose of glycitein. Most

Figure 3. Biotransformation of Genistein and Daidzein



Adapted from UK Committee on Toxicity *(3)*

of the human studies on the metabolism of genistein and daidzein as aglycones were obtained from literature based primarily on exposure to glycosides through soy products. Variations in metabolic pathways of isoflavones have been reported to occur as a result of differences in microflora, intestinal transit time, pH, or redox potential, factors that can be affected by diet, drugs, intestinal disease, surgery, and immune status (reviewed in Munro *et al.*, 2003*(178)*).

Metabolism of isoflavone glycosides begins with hydrolysis of the compounds to their respective aglycones, a step that is generally considered a prerequisite before the compounds can enter the systemic circulation. This initial deconjugation of the glycoside occurs mostly in the large intestine, but also in the mouth, stomach, and small intestine (reviewed in Larkin *et al.*, 2008 *(1)*).**[In accordance with well-understood principles of absorption, genistin and daidzin in soy products will not be readily absorbed because their high water solubility prevents passage through the lipid bi-layers of enterocytes. Also in agreement with theory is a prolonged t_{max} (time to C_{max}, indicating that the glucosides must first traverse the small intestine and reach the large intestine before bacterial flora deconjugation to genistein and daidzein, which are insoluble in water but soluble in lipids. The lipid solubility of genistein and daidzein facilitates their absorption in the large intestine.]** Because glycosides are relatively quickly deconjugated in the gut to form the active aglycones, exposure to a particular isoflavone is theoretically the sum of the aglycone and respective glycoside compound concentrations converted on the basis of molecular weight *(3; 5; 6)*.

Microbes in the intestinal tract catalyze reduction, demethlyation, and ring fission reactions to produce unconjugated aglycones and their metabolites, which are then absorbed and transported to the liver. Prior to entering the systemic circulation, these compounds are hydroxylated by P450s or conjugated with glucuronic acid by uridine diphosphate (UDP)-glucuronosyltransferase (UDPGT, UGT); a much smaller amount is conjugated to sulfate by sulfotransferase (SULT) enzymes *(3; 179; 180)* to form the more water-soluble isoflavone glucuronides and sulfates that are excreted in urine. In volunteers given an isoflavone aglycone formulation providing genistein doses of 2–16 mg/kg bw, ~8–18% of the genistein dose was excreted in urine as genistein conjugates within 24 hours *(153; 168)*, and less than 0.3% of the dose was excreted as free genistein *(168)*. Conjugation occurs in the liver, but at least for genistein, appears to occur to a significant degree in intestinal cells prior to transport to the liver. Human pharmacokinetic and bioavailability studies include plasma/serum or urine measurements of isoflavones and do not permit direst assessment of the contribution of intestine, liver, or other organs involved in isoflavone metabolism *(1)*

Most isoflavones in the circulation are present in conjugated form. In individuals who ingested a soy supplement consisting of isoflavone glycosides, glucuronides represented 69–98% of circulating genistein and 40–62% of circulating daidzein *(77)*. In the same study, sulfate conjugates represented 4% of circulating daidzein compounds in 1 man but were not detected in a female subject. Genistein sulfates were not detected. Setchell *et al.*, 2001 *(76)* reported mean steady-state circulating unconjugated isoflavones at 2.7% for daidzein and 1.6% for genistein when the compounds were taken as aglycones or glycosides.In studies where humans were exposed to genistein alone or in combination with other isoflavone aglycones (calculated as genistein doses of 1–16 mg/kg bw), most of the genistein was present in plasma in conjugated form *(76; 153; 168)*; free genistein represented 1–3% of total plasma genistein levels. One review reported that ~10% of isoflavonoids are circulated in plasma unconjugated *(108)*. Also, isoflavones can bind to plasma or serum proteins such as albumin *(181)*. The conjugated isoflavones can be transported through systemic circulation to tissues and eventually excreted via the kidneys or secreted in bile and returned to the intestine *(1)*. Upon return to the intestine, they are deconjugated by bacteria possessing β-glucuronidase or arylsulfatase activity. The metabolites may be reabsorbed and returned to the liver via the portal vein for reconjugation and additional enterohepatic circulation or renal excretion *(1)*.

A number of studies report that the shape of the plasma appearance and disappearance curves show early peaks prior to obtaining C_{max} *(76; 148; 151; 166)*. For example, Zubik *et al.*, 2003 *(152)* and Kano *et al.*, 2006 *(148)* noted that plasma concentrations of genistein and daidzein peaked at 1-2 hours and again at 4-8 hours following ingestion as aglycones or glucosides. This biphasic patterns are consistent with enterohepatic circulation or no enterohepatic circrulation but uptake in the small intestine as well as the large intestine (discussed in *(166)*). This early peak also suggest that hydrolysis and initial absorption occur readily in the duodenum and proximal jejunum following ingestion *(1)*.

Setchell *et al.*, 2001 *(76)* reported a small rise in the plasma concentration of daidzein after the administration of glycitin, but based on the overall plasma profiles there appeared to be negligible biotransformation of glycitin, other than initial hydrolysis of the glycosidic group. Demethoxylation to daidzein was a minor biotransformation pathway.

Bursztyka *et al.*, 2008 *(182)* compared the metabolism of genistein in rats and humans using liver microsome and hepatocytes. Human and rat liver microsomes and cryopreserved hepatocytes were incubated with

[^{14}C] genistein. Metabolite profiling was obtained using an HPLC system and identification was based on their retention times as compared with those of authentic standards and on LC–MS (ESI-MS/MS) or NMR analyses. In liver microsomes of both species, three hydroxylated metabolites were produced: 8-hydroxygenistein (8-OH), 6-hydroxygenistein (6-OH), and 3'-hydroxygenistein (3'-OH). Similarly, the same glucuronide and sulfate conjugates were produced in rat and human cryopreserved hepatocytes (genistein 4'-*O*-sulfate 7-*O*-glucuronide, genistein 7-*O*-glucuronide, genistein 4'-*O*-glucuronide, genistein 7-*O*-sulfate and genistein 4'-*O*-sulfate), with genistein 7-*O*-glucuronide identified as the major metabolite. In rat liver microsomes, the main metabolite produce was 3'-OH; however, in humans 3'-OH and 8-OH were produced in the same range.

UGT, SULT, and CYP Isoforms

A number of studies, summarized in **Table 35**, have characterized the UGT, SULT, or cytochrome *P450* (CYP) isoforms involved in the metabolism of genistein, daidzein, and glycitein [77; 183-187].

Doerge *et al.*, 2000 *(77)* characterized the enzymatic basis for isoflavone conjugates using microsomal recombinant human UGT isoforms (1A1, 1A4, 1A6, 1A7, 1A9, and 1A10), recombinant human SULT isoforms (1A1*2, 2A1, 1E, 1A2*1, 1A3), and by human tissue microsomes prepared from the liver, kidney, and colon. For genistein, the relative activity of the recombinant UGT isoforms was 1A10 > 1A9 > 1A1 > 1A6 > 1A7 >> 1A4. **[For 1A4, K_{cat} was < 0.03 and a K_m value was not presented.]** For daidzein, the UGT isoform activity decreased in the order of 1A9 > 1A1 > 1A4 >> 1A10=1A6=1A7 **[For 1A10, 1A6, 1A7, K_{cat} values were < 0.13 and K_m values were not presented].** For both genistein and daidzein, formation of the 7-glucuronide was favored over the 4'-isomer. UGT 1A9 and 1A1 displayed 2- to 3-fold higher substrate activity for daidzein compared to genistein. The UGT 1A10 isoform, which is present in colon, gastric, and biliary epithelium but not in liver, was observed to have the highest activity and specificity for genistein. No activity was observed for daidzein and any of the recombinant human SULTs tested. **[A daidzein sulfate metabolite was identified and a sulfate-glucuronide diconjugate was possibly detected in human volunteers after ingestion of a high-dose soy nutritional supplement, suggesting the participation of other SULT isoforms *in vivo*.]** For genistein, the activity for recombinant SULTs decreased in the order of 1A1*2 > 2A1 > 1E >> 1A2*1=1A3. Results from the glucuronidation of genistein in human tissue microsomes showed activity decreased in the order of kidney > colon > liver. The glucuronidataion of daidzein was similar in the kidney and liver but no activity was observed in colon microsomes. The relative acitivity of genistein to daidzein, based on k_{cat}/k_m ratio of genistein-7-glucuronide (G7) to daidzein-7- glucuronide (D7), were similar in the liver (0.85), 1.8-fold greater in the kidney, and much greater in the colon (k_{cat} for G7=56, k_{cat} for D7=< 0.08). Based on these observations, the study authors concluded that the intestine plays a major role in the glucuronidation of genistein and that most daidzein glucuronidation occurs in liver *(77)*.

Results presented in Doerge *et al.*, 2000 *(77)* related to the involvement of SULT1A1 and SULT2A1 in genistein metabolism are consistent with recent findings from Chen *et al.*, 2008 *(188)* that genistein can induce the protein and mRNA expression of SULT1A1 and SULT2A1 in HepG2 and Caco-2 cells.

Liu *et al.*, 2007 *(184)* characterized UGT isoforms responsible for the metabolism of genistein in intact Caco-2 cells and cell lysates using short interfering RNA ("silencing RNA", siRNA). They found that a number of well-expressed UGT isoforms in Caco-2 cells, UGT1A1, UGT1A3, UGT1A6, and UGT2B7, were involved in the metabolism of genistein.

Table 35. UGT, SULT, and CYP Isoforms Involved in the Metabolism of Genistein, Daidzein, and Glycitein

Isoflavone	Isoforms					
	UGT		SULT		CYP	
Genistein	Activity reported	•UGT1A1 [a,b,d,f] •1A3 [a,f] •1A6 [a,b,d,f] •1A7 [a,b] •1A8 [a,d] •1A9 [a,b,d] •1A10 [a,b,d] •2B7 [f] •2B15 [f]	Activity observed [b]	•SULT1A1*2 •2A1 •1E	Activity observed [e]	•CYP1A1 •1A2 •1B1 •3A4 •2E1h
	No activity reported	•1A4 [a,d,f] •2B4 [f] •2B7 [d] •2B17 [d] •2B17 [f]	No activity observed [b]	•1A2*1 •1A3	No activity observed [e,i]	•2B6 •2D6 •2E1
Daidzein	Activity reported	•UGT1A1 [a,b,d] •1A3 [c] •1A4 [b] •1A8 [a,d] •1A9 [a,b,c,d] •1A10 [a,d]	No activity observed [b]	•SULT1A1*2 •2A1 •1E •1A2*1 •1A3	Activity observed [e]	•CYP1A1 •1A2 •1B1 •3A4
	Very minimal activity reported [d,g]	•1A4 •1A6 •2B7 •2B15			No activity observed [e,i]	•2B6 •2D6 •2E1
Glycitein	Activity reported a	•UGT1A1 •1A3 •1A7 •1A8 •1A9 •1A10				
	Very minimal activity reported [d,g]	•1A4 •1A6 •2B4 •2B7 •2B15				

[a] Tang et al., 2009 *(187)*, [b] Doerge et al., 2000 *(77)*, [c] Chen et al., 2008*(185)*, [d] Pritchett et al., 2008 *(186)*, [e] Atherton et al., 2006 *(183)*; [f] Liu et al., 2007 *(184)*.
[g] In recombinant cells expressing human UGTs.
[h] In human liver microsomes.
In CYP2E1 supersomes.

The UGT1A1 isoform glucuronidated genistein the fastest followed by UGT1A3 (35% of that of UGT1A1), UGT2B7 (16%), UGT1A6 (15%), and UGT2B17 (4%). Chen *et al.*, 2008 *(185)* reported that daidzein was a substrate for both recombinant human UGT1A3 and UGT1A9.

Pritchett *et al.*, 2008 *(186)* characterized UGTs involved in the glucuronidation of genistein and daidzein using human liver microsomes characterized for specific UGT activities (UGT1A1 with 17α-ethynyloestradiol, UGT1A9 with propofol, UGT1A4 with trifluoroperazine) or commercially available microsome preparations from recombinant cells expressing UGT isoforms (1A1, 1A4, 1A6, 1A8, 1A9, 1A10(a), 1A10(b), 2B7, 2B15; 1A10(a) was a Gentest Supersome and 1A10(b) was a Panvera baculosome). Statistically significant linear correlations were observed between the rates of generation of the major glucuronide of both genistein and daidzein and the measured activities of UGT1A1, UGT1A9, but not UGT1A4. In the recombinant cell systems, UGTs 1A9, 1A10(b), 1A1, and 1A8 catalysed the glucuronidation of both genistein and daidzein. UGT1A10(a), purchased from Gentest, only catalysed genistein glucuronidation. No glucuronidation activity towards either isoflavone was observed for 1A4, 2B7, and 2B15.

Tang *et al.*, 2009 *(187)* characterized the isoform specific glucuronidation profiles for genistein, daidzein, and glycitein using 12 recombinant human UGTs (1A1, 1A3, 1A4, 1A6, 1A7, 1A8, 1A9, 1A10, 2B4, 2B7, 2B15, 2B17) and human intestinal and liver microsomes. Three concentrations of each isoflavone were incubated for 1 hour with a panel of BD Supersomes expressing the 12 human UGT isoforms. This provided a fingerprint of the major isoforms responsible for metabolizing each isoflavone at "low," "medium," and "high" concentrations. The authors then made predictions about tissue specific glucuronidation patterns based on the fingerprint results and the expression patterns of the UGT isoforms in liver and intestine. These predictions were tested by incubating the isoflavones with human liver and intestinal microsomes. Each isoflavone had a distinct metabolic fingerprint that was dependent on isoflavone structure and concentration. While the top isoforms differed based on which isoflavone and concentration was tested, UGT1A1, 1A8, 1A9, and 1A10 were always the top four for each isoflavone across concentrations (**Table 36**). **[Figure 3 of Tang et al., 2009 *(187)* shows minor activities for 1A3, 1A6, and 1A7 in the glucuronidation of genistein.]** In addition, UGT1A7 was shown to be important for the metabolism of glycitein. UGT1A1 and 1A9 were considered the most important isoforms at low concentrations of genistein, daidzein, and glycitein. At high concentrations,

Table 36. Relative Rates of UGT Enzymes at Low, Medium, and High Concentrations of Genistein, Daidzein, or Glycitein

Isoflavone	*Relative Rate Ranking (based on nmol/min/mg)*[a]			*Glucuronidation in Human Liver and Intestinal Microsomes*
	"Low" (2.5 µm)	*"Medium"*[b]	*"High" (35 µm)*	
Genistein	1A9 > 1A1 >> 1A8 > 1A10	1A8 > 1A9 >> 1A1 > 1A10	1A8 > 1A9 > 1A10 > 1A10	Intestine > Liver
Daidzein	1A9 > 1A1	1A9 > 1A1 >> 1A8	1A1 > 1A9 >> 1A8 > 1A10	Intestine > Liver
Glycitein[c]	1A1 >> 1A9 > 1A8 > 1A10	1A1 >> 1A8 > 1A10 > 1A9	1A8 > 1A1 > 1A10 >> 1A9	Intestine > Liver

[a] Relative tissue expression: UGT1A1 = liver > small intestine; 1A8 and 1A10 = small intestine > liver; 1A9 = liver.
[b] 10 µM for genistein and glycitein; 12 µM for daidzein.
[c] UGT1A7 was shown to be important for the metabolism of glycitein although it was not in the top four isoflorms at any concentration tested.
From Tang et al., 2009 *(187)*.

UGT1A8 displayed the highest enzymatic rate for genistein and glycitein (**Table 36**). The authors suggest that the presence of 4 isoforms with overlapping specifities results in a low potential for variations in isoflavone bioavailability based on genetic polymorphisms. Glucuronidation by human intestinal microsomes was faster compared to human liver microsomes for both genistein, daidzein, and glycitein, regardless of dose. For genistein concentrations of 2.5, 12.5 and 35 µM, the range of glucuronidation rates were ~3.5 to ~5 nmol/min/mg in human intestinal microsomes compared to ~1 to ~1.5 nmol/min/mg in human liver microsomes. For these same concentrations of daidzein, the range was ~2 to ~4 nmol/min/mg in human intestinal microsomes compared to ~0.75 to ~2 nmol/min/mg in human liver microsomes. For glycitein, the range was ~5 to ~10 nmol/min/mg in human intestinal microsomes compared to ~2.75 to ~3 nmol/min/mg in human liver microsomes.

It is important to distinguish between UGTs and SULTs that are *capable* of conjugating isoflavone aglycones and those that are *quantitatively important* in the disposition of the compounds. The latter requires knowledge of the relative abundance of each isoform in tissues most important in determining the systemic availability of isoflavones–intestine and liver. High quality data to address relative abundance are not available. Moreover, for the purposes of evaluating the disposition of isoflavones in soy formula in infants, tissue specific ontogeny data for each of the involved enzymes are needed. Such ontogeny data are largely limited to the liver *(189)*.

There is also evidence that cytochrome P450 (CYP) may be involved in the metabolism of isoflavones. Unidentified metabolites considered to be hydrolysis products have been detected following *in vitro* incubation of genistein with human recombinant CYP1A1, 1A2, 1B1, 2E1, or 3A4 isoforms (reviewed in *(3)*). Since the UK Committee on Toxicity evaluation, additional support for CYP involvement in isoflavone metabolism has appeared in the published literature. For example, Atherton *et al.*, 2006 *(183)* investigated CYP-mediated metabolism of genistein and daidzein using human liver microsomes characterised for specific CYP activities or commercially available microsome preparations from recombinant cells expressing CYP isoforms (1A1, 1A2, 1B1, 2B6, 2D6, 2E1, 3A4, Gentest Supersomes). For both genistein and daidzein, three major metabolites of daidzein and genistein were detected by HPLC analysis following incubation with human liver microsomes. The retention time on HPLC analysis was consistent with hydroxylated metabolites. Because the appearance of these metabolites was NADPH-dependent, their formation was consistent with catalysis by CYP. In addition, the liver preparations with the lowest rates of ethoxyresorufin *O*-dealkylation, indicating CYP1A2 activity, generated the lowest quantities of genistein and daidzein metabolites. CYP2E1 was implicated in the metabolism of genistein and daidzein in studies of chemical inhibitors of specific CYP activities in humal liver microsomes, but not in the screen of CYP Supersomes. Recombinant CYP1A1, 1A2, 1B1, and to a lesser degree, 3A4, generated metabolites of both genistein and daidzein. Overall, the authors concluded that CYP1A2 is a major contributor to the metabolism of genistein and daidzein in the liver and that extra-hepatic metabolism may occur through the involvement of CYP1A1 and 1B1 isoforms.

Equol "producers"

The metabolic profile of daidzein varies among individuals. Some individuals produce little or no equol or *O*-desmethylangolensin (ODMA). A growing literature exists on factors that may contribute to equol and/or ODMA production status, i.e., microbial factors, dietary consumption, lifestyles, anthropmetric factors *(190)*, as well as possible phenotypic differences between equol or ODMA producers and non-producers in relation to hormone levels and breast density *(190; 191)*. In particular, characterizing factors

that distinguish individuals who metabolize equol from daidzein ("producers") from "nonproducers" is an active area of research in understanding the clinical effects of isoflavone and soy exposure. Equol has a higher estrogenic potency compared to daidzein and inter-individual differences in the abilitity to produce equol is suggested as a contributing factor in variability in individual biological response to soy *(142)*.

Approximately 30-50% of individuals are able to convert daidzein to equol *(106; 172; 173; 190; 191)*. Setchell *et al.*, 2003 *(173)* reported that 3 of 8 subjects administered [^{13}C]daidzein converted this isoflavone to [^{13}C]equol. Serum concentrations of [^{13}C]equol peaked 24 hours after administration of [^{13}C]daidzein and equol was detected in 4 seperate collections from these women indicating they consistently produced equol. Half-lives for equol formation were reported at 2–4 hours in adults eating soy foods *(164; 165)*. A review by Price and Fenwick *(192)* reported that most equol was excreted as the glucuronide, with smaller amounts excreted as the sulfate conjugate in some humans.

Equol production appears to be enhanced following ingestion of diadzin compared to daidzein, and more prevalent production has been reported in individuals who consume a diet high in carbohydrate and fiber and low in dietary fat (reviewed in*(145)*). In a sample of postmenopausal women, Bolca *et al.*, 2007 *(172)* reported that women with higher intakes of polyunsaturated fatty acids and alcohol consumption were more likely to be strong equol producers. Fujimoto *et al.*, 2008*(193)* reported a significantly lower proportion of equol producers in men aged 10-19 compared to older men in a study of Japanese (10% of men aged 10-19 and 44% of men aged 50-59) and Korean (45% of men aged 10-19 and 65% of men aged 50-59) men. The equol producers consumed significantly more isoflavones than the non-producers.

Metabolic capacity of infants

In adults, approximately 30 to 50% of individuals are considered to be equol producers; however, young infants are generally considered less able to produce equol due to immaturity in gut microflora and/or underdeveloped metabolic capacity *(84; 142; 194)*. However, Hoey *et al.*, 2004 *(87)* reported detection of equol in the urine of 25% of 4-6 month old soy formula-fed infants and Setchell *et al.*, 1997 *(84)* measured detectable concentrations of equol in the plasma of 4 of 7 (57%) soy formula-fed infants (both of these studies are described in more detail below). While categorizing an individual as an "equol producer" is not simply a matter of detecting equol in urine or blood, the detection frequency of equol in infants overlaps with the percentage of adults considered to be equol producers.

Setchell *et al.*, 1997*(84)* reported the mean plasma concentration of equol measured in soy formula fed infants as ~2 µg/L. The plasma concentration is either lower *(97)* or similar to *(102; 103)* mean serum or plasma concentrations reported in Japanese women at delivery (**Table 9** in Chapter 1) and higher than average levels reported in typical European populations or vegans and vegetarians in the UK *(113)*.

Based on the most recent CDC data from NHANES, the geometric mean (10^{th}–90^{th} percentile) of equol detected in urine for people aged 6 years and older is 8.77 µg/L (<LOD–38.5) *(115)*. This value is approximately 3.7 to 5.2-fold higher than urinary concentrations of equol measured in infants by the CDC and reported in Cao *et al.*, 2009 *(94)*, including for infants fed soy formula who have much higher exposures to daidzein. Interestingly, urinary concentrations of equol did not differ appreciably in infants as a function of feeding method. The geometric mean for infants fed soy formula, cow's milk formula, and breast milk were 2.3 ng/ml, 2.4 ng/ml, and 1.7 ng/ml respectively.

The ability of infants to absorb and metabolize isoflavones was demonstrated by Hoey *et al.*, 2004 *(87)*. The study examined 60 infants and children, aged 4 months to 6 years, who were assigned to either a soy-formula or control group (n=30 in each) depending on whether they had consumed soy or cow-milk formula during infancy. Subjects were also divided into 4 groups according to age (4–6 months, 7–12 months, 1–3 years, and 3–7 years). Genistein, daidzein, and glycitein were present in the urine of all soy-fed infants in the 4–6-month age group, while *O*-desmethylangolensin and equol were detected in 75 and 25% of soy-fed infants, respectively. In contrast, isoflavonoids were very low or not detected in the 4–6-month control group. In subjects aged 7 months to 7 years who were given a soy challenge, ODMA was present in the urine of 75% of soy subjects and 50% of control subjects, while equol was present in the urine of 19% of soy subjects and 5% of control subjects. These percentages were similar between groups in the 3–7-year age group. Analysis of fecal samples showed that the total bacterial count was significantly higher in cow milk-fed infants than in the soy formula-fed infants. More specifically, the bacterial counts for *Bifidobacteria*, *Bacteroides*, and *Clostridia* were significantly higher in the cow milk-fed group, while the combined count for *Lactobacillus* + *Enterococcus* was not significantly different between groups. There were no significant differences between the soy-formula and control groups in fecal enzyme activities, pH, or short-chain fatty acid concentrations. The researchers concluded from the 4–6-month age group data that the isoflavones genistin, daidzin, and glycitin were well absorbed after hydrolysis in the gut because significant concentrations of their glucuronides were found in urine samples. They also deduced that the ability to hydrolyze glycosides to aglycones developed by the age of 4–6 months because such hydrolysis is required for absorption. Although a higher percentage of soy formula-fed infants than cow milk-fed infants of the younger age groups were able to convert daidzein to equol, the percentages were similar among the older children. Thus, the authors concluded that isoflavone exposure early in life has no lasting effect on isoflavone metabolism. They also noted the influence of formula type on the composition of the microflora present in the gut of infants.

Setchell *et al.*, 1997 *(84)* detected equol in the plasma of 4 of 7 (57%) of infants fed a soy-based formula. Equol was detected in 100% of infants fed a cow milk-based formula and with a peak level up to 2 orders of magnitude higher than in infants fed soy-based formula. This finding can be explained by the detection of equol in cow's milk. In contrast, equol was only detected in 1 of 7 (14%) breastfed infants. In a larger sample, Cao *et al.*, 2009 *(94)* were not able to detect equol in the blood (n=27) or saliva (n=120) of infants on a soy formula diet, although it was detectable in the urine of a small proportion, 6 of 124 (5%), of infants exclusively fed soy formula for at least two weeks. Equol was detected in a higher percentage, 22%, of infants fed a cow's milk-based formula. Differences in the blood-based measurements of equol in the Setchell *et al.*, 1997 *(84)* and Cao *et al.*, 2009 *(94)* studies likely reflect differences in limit of detection. The mean plasma concentration of equol measured in soy formula fed infants by Setchell *et al.*, 1997*(84)* was ~ 2 ug/L while the limit of detection in whole blood for equol in Cao *et al.*, xxYEAR*(94)* was 12 ng/ml **[12 ug/L]**.

2.1.1.3 Excretion

Most ingested genistein and daidzein is excreted in urine, with very little excreted in feces. Reviews by Nielsen and Williamson, 2007 *(143)* and Cassidy *et al.*, 2006 *(145)* include summary descriptive statistics for urinary and fecal excretion based on 16 human studies. **[Cassidy et al., 2006 *(145)* noted that these values have a high degree of uncertainty because there was considerable variability in estimates between individual studies.]** Following ingestion of the aglycone, the average urinary

recovery of genistein was 11% (range of 8.3-18) and for daidzein it was 34% (range of 26-50%). These values were generally similar to reported mean recoveries of genistein and daidzein following ingestion of the glycosides genistin (19-20%; range of 5.3-39%) and daidzin (36 and 50%; range of 15-62%). The percentage excreted in feces was much lower for both genistein (average of 3%, range of 0.5 to 8.6%) and daidzein (3.7%; range of 0.6 to 5.5%). These relative excretion patterns of isoflavones in urine and feces are consistent with other estimates of ~30% in urine and 1–4% in feces. (*(16)* and reviewed in *(3; 15)*). These fecal excretion data are in contrast to experimental animal data *(195)*, which show fecal excretion of ^{14}C-genistein and/or derivatives at 30–36% of dose. Relative to the amount ingested, these proportional recoveries are considered low suggesting bacterial degradation, non-absorption of a significant amount of ingested isoflavone, the existence of uncharacterized metabolites, and bilary excretion as a limiting factor to systemic availability (*(153)*; Hendrich *et al.*, 1998; Kulling *et al.*, 2000; Xu *et al.*, 1994; Lampe *et al.* 1998 as reviewed in Larkin *et al.*, 2008 *(1)*). **[A strong possibility must be entertained that some of the material escaped detection due to bacterial degradation. Therefore, fecal excretion of genistein and/or derivatives is almost certainly much higher than indicated.]**

Isoflavones are excreted in urine mostly as glucuronides, with much lesser excretion as sulfates and mean excretion rates for genistein and daidzein have been reported to peak 6-12 hours or 8-12 hours after intake (Adlercreutz *et al.*, 1993; Watanabe *et al.*, 1998; King and Bursill 1998; and Lu *et al.*, 1995 as reviewed in Larkin *et al.*, 2008 *(1)*). Lu *et al.*, 1998 *(164)* reported that total urinary excretion consisted of 1% aglycones and 99% glucuronidated metabolites. Bloedon *et al.*, 2002 reported that following ingestion of isoflavone mixtures containing ≥70% aglycones to postmenopausal women, <0.3% of the dose was excreted as free genistein or daidzein *(168)*. Chen *et al.*, 2007*(196)* measured urine samples collected from 8 Asian and British people before and after a 5-week period of consuming a soy supplement that contained 98% acetyl glucoside isoflavones. The concentrations of free isoflavones ranged from non-detectable to 12% of total for genistein and 10% of total for daidzein. Isoflavone glucuronides were present in the highest concentrations and the sulfate conjugates were detected in urine at higher concentrations than those of the free isoflavones but with considerable variability amoung subjects *(196)*.

In feces, isoflavones are primarily excreted in the unconjugated form with less than 10% being conjugated (Adlercreutz *et al.*, 1995 as reviewed in Larkin *et al.*, 2008 *(1)*). Mean *in vitro* fecal degradation half-lives for 14 volunteers were reported at ~8.9 hours for genistein and ~15.7 hours for daidzein *(157; 197)*. The majority of fecal isoflavones are recovered 2–3 days following ingestion (reviewed in *(3; 173)*).

The bulk of urinary excretion of daidzein and genistein occurs within 24 hours of isoflavone ingestion (Lu *et al.*, 1995; Setchell *et al.*, 2003 as reviewed in Larkin *et al.*, 2008 *(1)*). Lu *et al.*, 1998 *(164)* reported peak urinary excretion in volunteers who drank soy milk occurred at 8–10 hours, with 95% of excretion occurring within 24 hours. A constant elimination rate has been reported between 11 to 35 hours after food consumption (King and Bursill 1998 as reviewed in Larkin *et al.*, 2008 *(1)*). Consistent with plasma pharmacokinetic data that suggest enterohepatic circulation, genistein and daidzein have been reported to show multiple peaks during excretion (Watanabe *et al.*, 1998 as reviewed in Larkin *et al.*, 2008 *(1)*).

Urinary concentrations of daidzein are typically higher than genistein concentrations following consumption of soy foods, suggesting to some greater absorption of ingested daidzein compared to genistein (reviewed in *(1; 142)*). Proposed explanations for the apparent enhanced elimination

of daidzein compared to genistein include it having a lower molecular weight and greater water solubility (Xu *et al.*, 1994 as reviewed in Larkin *et al.*, 2008 *(1)*). King and Bursill 1998 suggested the lower hydrophilicity of genistein may promote its excretion in bile (reviewed in Larkin *et al.*, 2008 *(1)*). It is reported that administration of "probiotics", dietary supplements of live bacteria or yeasts, can reduce the urinary output of isoflavones *(198)*.

Urinary excretion in infants, children, and adults

Cruz *et al.*, 1994 *(199)* measured urinary excretion of genistein, daidzein, and equol in a study of 33 four-month old male infants. Twelve of the infants were fed breastmilk, 8 were fed a cow milk-based formula, and 13 were fed a soy milk-based formula, either containing no cholesterol or modified to contain cholesterol. Based on Figure 3 in the publication, urinary levels of each isoflavone were very low in breastfed infants. The median values of genistein, daidzein, and equol in infants fed a cow milk-based formula (genistein, ~30 µg/L; daidzein, ~40 µg/L, and equol, ~10 µg/L) appear to be similar to the geometric mean reported by the CDC for adults aged 20 to 60+ summarized in **Table 15** in Chapter 1 (genistein, 31.3–24.1 µg/L; daidzein,44.8–63.7 µg/L; and equol 8.54–7.52 µg/L) *(115)*. Urinary concentrations of genistein and daidzein were much higher in infants fed the typical and modified soy milk-based formulas (genistein, ~300 to ~375 µg/L; daidzein, ~250 to ~700 µg/L).

Setchell *et al.*, 1998 *(6)* compared the urinary concentrations of genistein and daidzein reported in Cruz *et al.*, 1994 *(199)* to levels reported in adults and concluded that the urinary concentrations in infants were slightly lower than urinary values of adults consuming a similar daily intake of isoflavones which could indicate poor renal clearance in early life (Frank *et al.*, 1995, Setchell *et al.*, 1984, Morton *et al.*, 1994, Lu *et al.*, 1995, Hutchins *et al.*, 1995, and Xu *et al.*, 1994 as reviewed in Setchell *et al.*, 1998 *(6)*).

Irvine *et al.*, *(91)* measured urinary excretion of genistein and daidzein in 4 infants fed soy formula. The soy formulas contained genistein equivalents at 81–92 mg/kg formula and daidzein equivalents at 44–55 mg/kg formula as determined by HPLC. Based on measured isoflavone levels, recommendations by formula manufacturers, and infant weights, the authors estimated that the infants received isoflavones 2.9–3.8 mg/kg bw/day from 2 to 16 weeks of age. Once per week until 8 weeks of age and once every 2 weeks up to 16 weeks of age, 3–5 used diapers not containing feces were collected over a 24-hour period. Urine was diluted with water, squeezed from diapers, and pooled over 24 hours. Samples were hydrolyzed, methanol-extracted, and analyzed by HPLC. Recovery of isoflavones from diapers was verified. Data were presented as mean ± SEM and analyzed by ANOVA. Average concentrations of isoflavones in urine were daidzein equivalents 2.9 ± 0.3 mg/L and genistein equivalents 1.5 ± 0.2 mg/L. Mean percentages of daily isoflavone intake detected in urine and normalized for creatinine levels were daidzein equivalents 38 ± 4% and genistein equivalents 13 ± 3%. Percent excretion was not affected by age. Isoflavone excretion rates varied little between infants and ranged from 0.37 ± 0.03 to 0.58 ± 0.06 mg/kg bw/day for daidzein and from 0.15 ± 0.03 to 0.32 ± 0.04 mg/kg bw/day for genistein. Age did not affect excretion rate. Urine from 25 infants fed dairy-based formula was also analyzed, and no isoflavones were detected **[detection limit not specified]**.

In addition to the studies described above, several recent studies from a research group associated with the Cancer Research Center of Hawaii have been published on elimination patterns of isoflavones in infants, children, and adults. This research group uses information on urinary isoflavone excretion rate (UIER) to draw conclusions on isoflavone bioavailability, i.e., higher excretion rates correspond

to higher bioavailability. The authors use urinary isoflavone analysis as a surrogate for measuring systemic isoflavone exposure based on the argument that UIER strongly correlates with circulating isoflavone levels when the timing of collection is correctly considered and when creatinine-based urinary values are correctly converted to time-based values taking into account BW, sex and age (see Franke *et al.*, 2006 *(95)* for a detailed presentation of this argument). The research group expresses urinary isoflavone excretion (UIE) relative to time and not creatinine based on the rationale that the latter is heavily influenced by muscle mass which varies as a function of age, gender, and body weight. UIER patterns in these studies are summarized in **Table 37**.

Franke *et al.*, 2006 *(95)*, supported by the Solae Company and the NCI, compared isoflavones patterns in the urine and plasma of tofu-fed infants (aged 9–25 months) and breastfed infants (2-45 weeks of age) and their mother milk and urine following maternal consumption of a soy protein beverage. Three infants consumed a tofu sample with an average of 7.4 mg isoflavones that resulted in an average (± SD) dose 0.694 ± 0.42 mg/kg. Sixteen mothers drank a soy protein beverage once daily for 2 - 4 days. Each beverage contained ~ 55 mg isoflavones (total daidzein:genistein:glycitein=1:1:0.1) which were present mostly (~82%) as glucosides and malonylglucosides and resulted in an average body weight adjusted dose of 1.01 ± 0.03 mg/kg. Samples of mother's urine (n=16), breastmilk (n=16), infant urine (n=13), and infant plasma (n=11) were collected in the afternoon following maternal consumption of the soy beverage in the early morning. Daidzein, genistein, glycitein, equol, dihydrodaidzein, dihydrogenistein, and *O*-desmethylangolensin were measured in breast milk, maternal urine, infant urine, and infant plasma by HPLC with photodiode array detection followed by electrospray ionization ion trap mass spectrometry. Results of this study related to urine elimination are presented here and data on infant and maternal plasma and breast milk concentrations at baseline and following soy food consumption are presented in Chapter 1.

Baseline urines were available for seven mother-infant pairs. Based on these samples, statistically significant increases in mean (± SEM) isoflavone excretion rates following the mothers ingestion of the soy protein beverage were observed in mother's urine (18.4 ± 13.0 verus 135 ± 26.0 nmol/mg creatinine) and urine of breastfed infants (29.8 ± 11.6 verus 111.6 ± 18.9 nmol/mg creatinine). Inclusion of study participants who only donated specimans following the intervention (9 additional mothers and 5 additional breastfed infants) resulted in slightly higher but similar means: 157.1 ± 18.5 nmol/mg creatinine for mother's urine and 186.1 ± 25.1 nmol/mg creatinine for urine of breast fed infants. Compared to either the mothers fed a soy beverage or their breastfed infants, the tofu-fed infants had much higher average levels of isoflavones in urine (229 nmol/mg creatinine; median: 145nmol/mg creatinine; range: 61–482 nmol/mg creatinine).

For each group, the authors also calculated a body weight- and time-adjusted urinary isoflavone excretion (UIE) value that was further adjusted for dose per kg body weight [$(nmol \cdot h^{-1} \cdot kg^{-1})$/ $(mg \cdot kg^{-1} \cdot d^{-1})$. Dose per body weight corrected UIE in breastfed infants was 18.4 percent of the maternal value (25.5 ± 3.3 versus 136.5 ± 16.3), a statistically significant difference. In contrast, the UIE/dose was 24% higher in tofu-fed infants than the maternal value (169.4 ± 32.1 versus 136.5); however, this increase was not statistically significant. The authors conclude that more isoflavones appear in children than in adults after adjustment for isoflavone intake. **[In Halm et al., 2007 *(200)*, the authors cite this study as showing that "relative to their mothers, urinary IFL excretion rate (UIER) was much lower in infants breast fed from soya-consuming mothers, but higher in babies eating tofu."]**

Halm *et al.*, 2007 *(200)*, supported by the Revival Company and the NCI, compared isoflavone excretion rates in 19 children and adolescents (ages 3 to 17) and 18 adults following consumption of a body weight-adjusted dose of soya nuts of 15g per 54.4 kg body weight (equivalent to 0.615 ± 0·036 SD mg total isoflavones/kg body weight) followed by a 12 hour urine collection. Daidzein, genistein, glycitein, equol, dihydrodaidzein, dihydrogenistein, and *O*-desmethylangolensin were measured by HPLC with photodiode array detection followed by electrospray ionization ion trap mass spectrometry. Isoflavone content in baseline samples was subtracted from the isoflavone content in the 12-hour urine collection sample to adjust for background.

Urinary excretion rates were significantly higher in children compared to adults for daidzein (+ 39%; 30.5 versus 22.0 nmol/h per kg), genistein (+ 44%; 12.0 versus 8.5 nmol/h per kg), all non-metabolites (daidzein + genistein + glycitein; + 41%; 44.8 versus 32.7 nmol/h per kg) and total isoflavonoids (+ 32%; 51.1 versus 39.7 nmol/h per kg). Urinary excretion rates did not differ for glycitein, equol, dihydrodaidzein, dihydrogenistein, and *O*-desmethylangolensin, or all metabolites when considered together. These data are summarized in **Table 37**. There were no apparent differences in UIE when the 19 children were divided into two groups, ages 3-9 (n=13) and 12–17 (n=6). The authors interpret these findings as indicating that isoflavones are more bioavailable in children than adults. They hypothesize that greater isoflavone uptake in children could be due to their gut flora that is able to hydrolyse isoflavonoids to the bioavailable aglycone efficiently but does not degrade the aglycones as fast as adults.

Halm *et al.*, 2008 *(201)*, supported by the Revival Company and the NCI, conducted a study to compare the effects of oral antibiotic use on UIER in children. The authors conducted this study in part to follow-up on their observation of higher isoflavone bioavailability in one healthy adult subject following a 1-day treatment with oral neomycin and erythromycin. That finding was hypothesized to be due to a mild reduction of the gut flora that is responsible for the degradation of isoflavones. In the current study, isoflavone excretion was measured in eleven children aged 4 to 7 at two time points (1) during oral antibiotic use, and (2) several weeks after antibiotic treatment when the children were healthy. At each time point urine samples were collected before and after consumption of 15 g soy nuts/54.4 kg body weight. This resulted in the following doses: total isoflavones=429.3 µg aglycone units/kg bw; total daidzein=194.3 µg aglycone units/kg bw; total genistein=227.1 µg aglycone units/kg bw; and total glycitein=7.9 µg aglycone units/kg bw. The post-soy nut consumption urine sample represented all overnight urine voids, a ~ 12-hour collection period. All children were on antibiotics for at least 3 days prior to the first soy nut challenge, and if possible, the soy challenge was conducted on the second to last day of the therapy. Daidzein, genistein, glycitein, equol, dihydrodaidzein, dihydrogenistein, and *O*-desmethylangolensin were measured by HPLC with photodiode array detection followed by electrospray ionization ion trap mass spectrometry. Isoflavone content in baseline samples was subtracted from the isoflavone content in the overnight urine collection sample to adjust for background.

Average UIER (± SEM) was significantly decreased during oral antibiotic use versus when healthy for genistein (6.4 ± 1.0 versus 10.1 ± 1.6 nmol/h/kg), all non-metabolites (daidzein + genistein + glycitein; 27.5 ± 4.8 versus 36.2 ± 4.7 nmol/h/kg), and total isoflavones (29.4 ± 5.0 versus 38.8 ± 4.8 nmol/h/kg). Although the excretion rate of total isoflavones was significantly lower during antibiotic use, in 4 of the 11 children the total isoflavone UIER increased during antibiotic use. The authors did not observe any relationship between UIER and the specific type of antibiotic used, type of illness, age, gender, or ethnicity. In addition, 4 "non-producers" of desmethylangolensin produced the metabolite while

on antibiotics and 1 equol "producer" did not have a measurable urine concentration during antibiotic use. This latter finding was considered consistent with the involvement of several bacteria in daidzein metabolism. In addition, it suggest that labels of "producers" and "non-producers" for individuals may not hold over time and can be influenced by multiple factors.

Overall, the observed pattern of UIER is not consistent with the investigators previous observation of higher isoflavone bioavailability in one healthy adult subject following a 1-day treatment with antibiotics. The authors suggest that this discrepancy may be due to differences in gut flora between children and adults that can affect isoflavone absorption and degradation as well as health status and duration of antibiotic use (sick children receiving antibiotics for 5—14 days compared to a 1-day treatment in a healthy adult).

Franke *et al.*, 2008 *(202)*, supported by the Revival Company and the National Cancer Institute, conducted a pilot study to compare the effects of oral antibiotics on UIER in 7 children (average age of 12.2 years) and 12 adults (average age of 37.8 years) after soy consumption. Subjects provided a baseline and overnight urine sample before and after consuming soy nuts, once while on oral antibiotics and again at least 4 weeks later when subjects were healthy and no longer taking antibiotics. During oral antibiotic use versus when healthy, UIER in adults was significantly increased for daidzein (35.2 versus 18.9 nmol/h/kg), daidzein + genistein + glycitein [nonmetabolites (NM); 42.6 versus. 23.6 nmol/h/kg), and total isoflavonoids (daidzein + genistein + glycitein + dihydrodaidzein + dihydrogenistein + equol + O-desmethylangolensin) (51.5 versus 29.6 nmol/h/kg). In contrast, children showed significantly reduced UIER when on oral antibiotics versus when healthy for daidzein (36.3 versus. 46.8 nmol/h/kg), dihydrodaidzein (1.2 versus 3.0 nmol/h/kg), NM (46.3 versus 59.5 nmol/h/kg), all metabolites (1.0 versus 4.3 nmol/h/kg; based on dihydrodaidzein + dihydrogenistein + equol + O-desmethylangolensin, and total IFLs (48.2 versus. 63.8 nmol/h/kg). Significantly higher mean UIER for genistein (10 versus 4.4 nmol/h/kg), daidzein (46.8 versus 18.9 nmol/h/kg), glycitein (2.7 versus 0.3 nmol/h/kg), non-metabolites (59.5 versus 23.6 nmol/h/kg), and total isoflavones (63.8 versus 29.5 nmol/h/kg) were reported in healthy children compared to healthy adults. This finding led the authors to conclude that the apparent isoflavone bioavailability is higher in healthy children compared to healthy adults.

Franke *et al.*, 2008 *(175)*, supported by the Physicians Pharmaceuticals, Inc. and the NIH, compared the effects of oral antibiotics on isoflavone UIER in children (ages 4–17 years) and adults (≥18 years). A soy nut challenge and urine collection protocol was conducted twice in 16 children and 12 adults, initially when subjects were on oral antibiotic treatment and repeated at least 4 weeks later when subjects were healthy and no longer taking antibiotics. In addition, UIER following soy challenge was evaluated in another 37 children and 34 adults who were healthy and not taking oral antibiotics. The total numbers of subjects evaluated when healthy and not taking oral antibiotics were 53 children and 46 adults. The article notes that 5 children and 17 adults were given 15 g of soy nuts while other study participants were given a body weight adjusted amount of 15 g nuts per 54.4 kg body weight. The authors reported the results from all participants because exclusion of the non-body weight adjusted participants did not change the results significantly.

Franke *et al.*, 2008 *(175)* also included an intervention protocol in adults to assess the correlation between plasma and urine isoflavone values. Those results are also discussed in **Section 2.1.1.1** ("Correlations between isoflavone levels in plasma, urine, and other fluids or tissues") Eight men and six women consumed 10 g of soy nuts (~ 22.1 mg isoflavone aglycone equivalents). Blood samples were collected at

3-6 hours and 4-9 hours after soy intake. Each participant emptied their bladder shortly after the first blood draw and the urine sample for analysis was collected between the two blood draws. The authors did not adjust final urinary excretion rates for body weight because the body weights of study participants were similar. The correlation coefficient between isoflavone levels in urine and plasma was calculated using linear regression. Isoflavone levels in the two matrices were better correlated, r=0.93 (P<0.001) when area under the curve (AUC) for both plasma and urine (both time-based units) were used rather than UIER (a time-based measure, nmol/h) and plasma isoflavone levels at first collection (a non-time-based unit, nM). The correlation between UIER and plasma level at first collection was 0.68 (not statistically significant).

With respect to UIERs, this study generally replicated findings of two other studies conducted by this research group (**Table 37**). First, the authors found that healthy children have significantly higher UIERs compared to adults as was reported in Halm *et al.*, 2007 *(200)*. Results were statistically significant for daidzein (27.1 versus 19.3 nmol/h/kg), genistein (9.2 versus 6.1 nmol/h/kg), non-metabolites (36.8 versus 25.8 nmol/h/kg), and total isoflavones (39.6 versus 31.3 nmol/h/kg). Also, compared to when healthy, children taking oral antibiotics had significantly reduced UIERs as reported in Halm *et al.*, 2008 *(201)*. Results were statistically significant for daidzein (26.1 versus 32.7 nmol/h/kg), genistein (6.9 versus 11.3 nmol/h/kg), non-metabolites (34 versus 43.1 nmol/h/kg), and total isoflavones (35.6 versus 47.7 nmol/h/kg). The new UIER finding reported in Franke *et al.*, 2008 *(175)* was that the effect of oral antibiotics on UIER differs in children and adults. As described above and in **Table 37**, UIER decreased in children during treatment with oral antibiotics. In contrast, UIER was significantly increased in adults during antibiotic treatment for daidzein (35.2 versus 18.9 nmol/h/kg), genistein (P=0.56, 6.8 versus 4.4 nmol/h/kg), non-metabolites (42.6 versus 23.6 nmol/h/kg), and total isoflavones (51.5 versus 29.6 nmol/h/kg).

2.1.2 Experimental Animals

The majority of the literature on pharmacokinetics in experimental animals is based on studies of genistein and much less information is available for other isoflavones. In contrast to humans, who are exposed to genistein primarily through soy product intake, many of the toxicokinetic studies in experimental animal studies involved direct dosing with genistein, generally in the aglycone form. A relatively limited number of experimental animal studies examined serum or tissue levels of genistein and other isoflavones following consumption of soybean-based animal feeds. The results of these studies indicate that consumption of a soy-based diet results in detectable blood levels in adult animals that are directly consuming the diet as well as in fetuses exposed via the dam and in neonatal animals during lactation. Pharmacokinetic studies have been conducted in domestic animal species or livestock, e.g., cats, pigs, cows *(203-206)*. These studies were not considered to any significant degree in the expert panel evaluation because of the extensive pharmacokinetic literature available in laboratory animal species that are primarily used as the toxicological models for predicting potential health effects of genistein in humans, i.e., rodents and non-human primates. In addition, hepatic conjugation in cats is known to differ from other species such that they more readily form sulfates of phenolic compounds rather than glucuronides (reviewed in *(204)*).

2.1.2.1 Absorption

Studies that summarize genistein levels measured in rats or mice fed a phytoestrogens-free diet and dosed with genistein are summarized in **Table 38**. This table includes data for adult animals that were directly administered genistein in addition to information on fetuses and infant rodents whose dams were treated with genistein.

Table 37. UIER Patterns Following a Body Weight Adjusted Soy Challenge in (1) Children versus Adults, (2) Children During and After Treatment with Oral Antibiotics, and (3) Adults During and After Treatment with Oral Antibiotics

Isoflavone	*Children versus Adults (% change from adult)* [a,b,d]	*Oral Antibiotics versus when healthy in Children (% change from healthy)* [b,c,d]	*Oral Antibiotics versus when healthy in Adults (% change from healthy)* [b,d]
Daidzein	+40[a,b] to +148[d]	NS[c] to −20[b] to −22[d]	+87[b,d]
Genistein	+53[a,b] to +127[d]	NS to −36[c] to −40[b]	+54[b,d]
Glycitein	NS[a,b] to +800[d]	NS[b,c]	NS
Total Non-metabolites (Daidzein + Genistein + Glyciten)	+42[a,b] to +102[d]	−21[b] to −22[d] to −24[c]	+81[b,d]
Equol	NS	NS	NS
Desmethylangolensin (DMA)	NS	NS	NS
Dihydrodaidzein (DHDE)	NS	NS[b,c] to −59[d]	NS
Dihydrogenistein (DHGE)	NS	NS	NS
Total Metabolites (Equol + DMA + D + A + DHDE + DHGE)	NS	NS[b,c] to −77[d]	NS
Total Isoflavones	+26[b] to +170[d]	−24[c] to −25[b,d]	+74[b,d]

[a] n = 19 children and 18 adults from Halm et al., 2007 *(200)*.

[b] n = 53 children and 46 adults for children versus adult comparison, n = 16 children for oral antibiotic versus healthy comparison, and n = 12 adults for oral antibiotic versus healthy comparison from Franke et al., 2008 *(175)*.

[c] n = 11 children for oral antibiotic versus healthy comparison from Halm et al., 2008 *(201)*.

[d] 7 children and 12 adults from Franke et al., 2008 *(202)*.

NS = Not statistically significant difference.

Table 38. Blood Genistein Levels in Rodents Fed Phytoestrogen-Free Diets and Dosed with Genistein

Species and Strain	Age and Number of Animals	Route and Duration	Dose	Age or Sex	Serum Genistein, nM [ng/ml or µg/L]		Percent Aglycone	Reference
					Total	Aglycone		
Rat, Sprague Dawley	Dams and fetuses on GD 20 or 21 n=1 dam/litter (11–16 fetuses)/ group	Oral (gavage) Single treatment of dam on GD 20 or 21 Blood collected 2h after dosing	20 mg/kg bw	Dams	3540 **[956]**	270 **[73]**	8%	Doerge et al., 2001 *(208)*
				Fetuses	270 **[73]**[a]	80 **[22]**[a]	31%	
			34 mg/kg bw	Dams	5480 **[1480]**	290 **[78]**	5%	
				Fetuses	190 **[51]**	60 **[16]**	34%	
			75 mg/kg bw	Dams	4410 **[1191]**	780 **[211]**	18%	
				Fetuses	220 **[59]**	60 **[16]**	27%	
	Males and females, PND 1–2 n=2–3 pups from 2 different litter pools	Oral (diet) Pups exposed indirectly during gestation and lactation	Dams, 500 ppm (~50 mg/kg bw/d)		176 ± 307 **[48]**	47 **[13]**	53 **[27% by CERHR calculation]**	
Rat, Sprague Dawley	Dams and pups on PND 7 and 21 **[Number examined not reported]**	Oral (diet) Dams exposed during gestation and lactation	0	Dams	6 **[2]**[a]	6 **[2]**[a]	100%	Fritz et al., 1998 *(209)*
				PND 7	9 **[2]**	9 **[2]**	100%	
				PND 21	6 **[2]**[a]	6 **[2]**[a]	100%	
			25 ppm (~2.2 mg/kg bw/d)	Dams	40 **[11]**	9 **[2]**	23%	
				PND 7	86 **[23]**	16 **[4]**	19%	
				PND 21	54 **[15]**	18 **[5]**	33%	
			250 ppm (~22 mg/kg bw/d)	Dams	418 **[113]**	7 **[2]**	1.7%	
				PND 7	726 **[196]**	103 **[28]**	14%	
				PND 21	1810 **[489]**	120 **[32]**	6.6%	
Rat, Sprague Dawley	Females, PND 9 n=4	Oral (diet) 21 days starting at PND 70	750 ppm		2200 **[584]**	400 **[108]**	18.2%	Santell et al., 1997*(211)*
Rat, Sprague Dawley	Males, PND 70 n=8/group	Oral (diet) Exposed via dam during pregnancy and lactation, and then directly until PND 70	0 ppm		18 **[5]**[a]	0	0%	Fritz et al., 2002*(212)*
			25 ppm (~2.2 mg/kg bw/d)		167 **[14]**	6 **[2]**[a]	3.6%	
			250 ppm (~22 mg/kg bw/d)		1908 **[515]**	20 **[5]**	1.0%	
		Oral (diet) on PND 57–65 Gavage on PND 66–70	0		28 **[8]**[a]	6 **[2]**[a]	21.4%	
			250 ppm diet 22 mg/kg bw/d gavage[b]		1785 **[482]**	32 **[7]**	1.8%	
			1000 ppm diet 88 mg/kg bw/d gavage[b]		9640 **[2602]**	41 **[11]**	0.43%	

Table 38 (continued)

Species and Strain	*Age and Number of Animals*	*Route and Duration*	*Dose*	*Age or Sex*	*Serum Genistein, nM [ng/ml or µg/L]*		*Percent Aglycone*	*Reference*
					Total	*Aglycone*		
Rat, Sprague Dawley	Offspring, PND 21 and 140 Males and females (n = 5 – 6/group)	Oral (diet) Exposed via dam during pregnancy and lactation Offspring received dam's diet at weaning	0	M	< 10 **[< 3]**	–	1–5% all dose groups, both ages	Chang et al., 2000 *(210)*
				F	< 10 **[< 3]**			
			5 ppm **[~0.4–0.5 mg/kg bw/d]**	PND 21, M	22 **[6]**	–		
				PND 21, F	20 **[5]**			
				PND 140, M	60 **[16]**			
				PND 140, F	100 **[27]**			
			100 ppm **[~8–10 mg/kg bw/d]**	PND 21, F	270 **[73]**	–		
				PND 21, M	520 **[140]**			
				PND 140, M	590 **[159]**			
				PND 140, F	940 **[254]**			
			500 ppm **[~40–50 mg/kg bw/d]**	PND 21, M	2090 **[564]**	–		
				PND 21, F	1870 **[505]**			
				PND 140, M	6000 **[1620]**			
				PND 140, F	7940 **[2144]**			
Rat, Sprague Dawley	Adult female dams n = 4/group	Oral (diet) on GD 7 – PND 21	250 ppm		2100 **[567]**	30 **[8]**	1.4%	Holder et al., 1999 *(213)*
	Female offspring, PND 63 n = 10/group	Oral (diet) Exposed indirectly during gestation (from PND 7), through lactation (PND 21), then directly on PND 21 – 63	250 ppm		1310 **[354]**	38 **[10]**	2.9%	
			1250 ppm		5300 **[1431]**	150 **[40]**	2.8%	
	Adult Male and female n = 10/group; n = 7 – 10/group	Oral (diet), **[duration of treatment not clearly reported]**	25 ppm (2 mg/kg bw/d)		≤250 **[≤68]**[e]	–	–	
			250 ppm (20 mg/kg bw/d)	Male	1500 **[405]**	–	–	
				Female	2000 **[540]**			
			1250 ppm (100 mg/kg bw/d)	Male	6000 **[1620]**	–	–	
				Female	9000 **[2430]**			

Table 38 (continued)

Species and Strain	Age and Number of Animals	Route and Duration	Dose	Age or Sex	Serum Genistein, nM [ng/ml or µg/L]		Percent Aglycone	Reference
					Total	Aglycone		
Rat, Sprague Dawley	Females, 11 weeks n=2–8	Oral (diet) for 3 weeks, beginning at 8 weeks of age	0		49 **[13]**[a]	–	–	Cotroneo and Lamartiniere *(214)*
			250 ppm (~16 mg/kg bw/d)		1115 **[301]**	138 **[37]**[a]	12	
			1000 ppm		2031 **[548]**	446 **[120]**	23	
	Females, 11 weeks n=4–5	SC for 3 weeks, beginning at 8 weeks of age Blood collected 16–18h after last injection	0		4 **[1]**[a]	–	–	
			5 mg/kg bw/d		450 **[122]**	–	–	
			16.6 mg/kg bw/d		1380 **[373]**	662 **[179]**[a]	48	
			50 mg/kg bw/d		5090 **[1374]**[a]	2243 **[606]**[a]	44	
Rat, Sprague Dawley	Female, PND 21, 50 and 100 n=6–9	SC, single dose given at PND 21, 50, or 100 Blood collected 16–18h after injection	500 mg/kg bw	PND 21	5558 **[1501]**[a]	1956 **[528]**[a]	**[35]**	Cotroneo et al., 2001*(215)*
				PND 50	39 **[11]**	16 **[4]**	**[41]**	
				PND 100	13 **[4]**	6 **[2]**	**[46]**	
Mouse, CD-1	Males amd females, PND 1–5 n=3–8/sex/ time period	SC, PND 1–5 Blood collected between 0.5 and 24h following injection	50 mg/kg bw/d	Male	3800 **[1026]**	**~1400 [378]**	31	Doerge et al., 2002 *(216)*
				Female	6800 **[1836]**	6800 **[1836]**		
Mouse, CD-1	Female, PND 1–5 n=4–6/time point	Oral (gavage) Blood collected 0.5–48h after last dosing	37.5 mg/kg bw/d (administered dose in aglycone equivalents)		19200 **[5189]**, C_{max}	5600 **[1513]**, C_{max}	29% at C_{max}	Jefferson et al., 2009 *(217)*
	Female, PND 1–5 n=4–6/time point	Oral (gavage) Blood collected 0.5–48h after last dosing	37.5 mg/kg bw/d		~1000 **[270.2]**, C_{max}			

GD=gestational day, PND=postnatal day; SC=subcutaneous; M=male; F=female. Conversions to µg/L refer to genistein equivalents.

[a] Values assumed to be expressed in means ± variance **[undefined]**.

[b] Dietary and gavage treatment provided equivalent doses. [**Gavage doses said to be equivalent to dietary doses, which suggests that feed intake was about 36 g/rat. This estimate appears reasonable to the Expert Panel.]**

[c] Expressed as mean ± SEM.

[d] Expressed as mean ± SD.

[e] Values estimated from a graph by CERHR

In 2001, Whitten and Patisaul *(108)* published a comprehensive review of phytoestrogens that included a comparison of isoflavone absorption and excretion in several species of animals (**Table 39**). Some of their conclusions were that (1) estimates of genistein bioavailability in rats and mice, 10-20%, are similar to values reported for humans although the rodent data were mostly based on high dose treatment with genistein aglycone; (2) plasma C_{max} is reached in rats within 2 hours and in mice within 3–30 minutes after oral dosage; (3) oral genistein doses of 20–45 mg/kg, either single or repeated, produce C_{max} plasma concentrations of 2–11 μM in rats; (4) single oral genistein doses of 50–200 mg/kg produce C_{max} free plasma concentrations of 1–4 μM in mice. These doses are considerably higher than those reported to produce μM plasma levels in humans, but it is unclear whether this difference is due to species differences or differences based on the administered form, i.e., aglycone, dietary soy, supplements.

Less information is available for other isoflavones compared to genistein. Janning *et al.*, 2000 *(207)* characterized the toxicokinetics of daidzein in female DA/Han rats following iv (10 mg/kg) or gavage (10 or 100 mg/kg) treatment. The plasma elimination half-life was ~4.2 hours. The oral bioavailability of daidzein was higher at 10 mg/kg compared to 100 mg/kg, 9.7% verus 2.2%. Multiple peaks in plasma concentrations during the course of the study indicated considerable degree of enterohepatic circulation. Tissue concentrations in the liver, kidney, and the uterus were often higher than plasma levels indicating uptake and storage of isoflavones and metabolites into tissue.

Comparisons based on administered form

Similar to the literature for humans, studies in experimental animals are inconsistent on the comparative pharmacokinetics and bioavailability following administration of isoflavones as aglycones or as glucosides. A number of the recent studies are described below to illustrate this pattern.

Steensma *et al.*, 2006 *(224)* compared the pharmacokinetics of genistein in both its aglyconic and glycoside forms in rats following a single 15 mg/kg gavage administration of genistein and genistin. Rats were cannulated in the portal vein and blood samples were collected in the 7-hour period after dosing. The AUC and C_{max} for plasma genistein were ~ 3.7 and 6-times higher following administration of genistein compared to genistin. These results led the authors to conclude that bioavailability was greater following ingestion of the aglycone. In addition, the time to achieve C_{max} occurred fairly early after dosing and at the same time point for both compounds (t_{max} of 15 minutes) which led the authors to conclude that the initial hydrolysis of genistin to its aglycone genistein is relatively fast and not a major rate-limiting step for the absorbtion of genistin. The author's analyzed plasma from rats administered soy extract and found similar genistein bioavailability when compared to the rats treated with genistin suggesting that other isoflavones present in soy do not impact genistein bioavailability. This study also reported detecting low concentrations of genistin in plasma following oral administration of either genistin or the soy extract indicating intestinal absorption of the glycoside. These authors note this latter finding conflicts with studies in humans conducted by Setchel *et al.*, 2002 *(147)* that reported a lack of absorption of the glucoside following ingestion of genistin.The authors suggested the difference in study outcomes may be because Setchell *et al.*, 2002 collected blood via vein puncture whereas the rat blood was collected from the portal vein to minimize the contribution of liver metabolism. Plasma glucosides were also previously observed in mouse Allred *et al.*, 2005 *(225)*.

Kwon *et al.*, 2007 *(226)*, supported by the Korean Ministry of Health & Welfare, characterized the pharmacokinetics of genistein and genistein-7-glucoside following oral and IV administration in 8-week

old male Sprague-Dawley rats (n=5-6 per dose group). Following oral administration of 4, 20, or 40 mg/kg genistein, plasma samples were collected at 0, 0.25, 0.5, 1, 2, 3, 4, 6, 8, 12, and 24 hours. Other animals were orally dosed with 64 mg/kg genistein glucoside (equal to 40 mg/kg genistein in aglycone equivalents) and plasma samples were collected at 0, 2, 4, 6, 7, 8, 9, 10, 12, 14, 18, 24, 30, 36, and 48 hours. Following IV administration of 1 mg/kg genistein or 1.6 mg/kg genistein glucoside (equal to 1 mg/kg genistein in aglycone equivalents), plasma samples were collected at 0.033, 0.083, 0.167, 0.25, 0.5, 1, 2, 3, 4, 6, 8, and 12 hours. Plasma concentrations were analyzed by HPLC following enzymatic hydrolysis with *H. pomatia*. Pharmacokinetic data are summarized in **Table 40**. Compared to oral administration of 40 mg/kg genistein, the same oral dose in aglycone equivalents of genistein glucoside (64 mg/kg) resulted in a longer t_{max} (8 hours versus 2 hours), lower C_{max} (3764 ng/ml versus 4876 ng/ml), higher $AUC_{0-\infty}$ (51,221 ng h/ml versus 31,270 ng h/ml), and higher absolute bioavailability (48.7% versus 30.8%) compared to oral administration of genistein. The authors also compared the pharmacokinetic profile of genistein following oral administration of genistein with and without enzymatic hydrolysis. The C_{max} of plasma genistein without enzymatic treatment was 3.2% of the plasma C_{max} following enzymatic hydrolysis, indicating that 97% of plasma genistein was circulating as conjugated with β-glucuronide and sulfate. The overall conclusion from the authors was that the oral bioavailability of genistein glucoside is greater than the aglycone.

Sepehr *et al.*, 2007 *(227)* characterized the pharmacokinetics of three sources of isoflavones in 3-month old male and female Sprague-Dawley rats. Rats (n=4 per group) were orally gavaged one time with either 20 mg/kg of Novasoy™, a commercial supplement with a ratio of genistin, daidzin, and glycitin of 1.0:0.5:0.2, or mixtures of synthetic aglycones or glucosides designed to achieve the same isoflavone ratios. The 20 mg/kg mixture of synthetic aglycones contained 11.76 mg genistein, 5.88 mg daidzein, and 2.36 mg glycitein. The 32.4 mg/kg treatment with a mixture of synthetic glucosides was formulated to be equimolar to the mixture of synthetic aglycones and contained 19.09 mg genistin (equal to 11.76 mg genistein), 9.62 mg daidzin (equal to 5.88 mg daidzein), and 3.7 mg glycitin (equal to 2.36 mg glycitein). Animals were also treated by IV injection with mixtures of synthetic aglycones and glucosides in the same proportions used in the oral gavage treatment groups. Absorption was determined by comparing plasma AUC after IV administration with the plasma AUC after oral administration. Absolute bioavailability was also calculated. Bioavailability parameters for Novasoy™ were estimated by comparing AUC_{oral} post-Novasoy administration by AUC_{IV} post-glucoside IV injection. Plasma samples were collected 0, 10, 30 minutes, and 1, 2, 8, 24 and 48 hours post-oral gavage treatment or 0, 10, 30, 45 minutes and 1, 2, 3, 4, 8 hours post-IV dosing. Plasma isoflavones were measured by LC/MS following enzymatic hydrolysis of isoflavone conjugates with a mixture of glucuronidase and sulfatase from *Helix pomatia*. Pharmacokinetic parameters are summarized in **Table 41** Overall, bioavailability of genistein, daidzein, and glycitein was significantly higher following ingestion of Novasoy™ and the glucoside forms compared to the aglycones in both male and female rats.

Sepehr *et al.*, 2009 *(228)* characterized the pharmacokinetics of three sources of isoflavones in 20-month old $F_3$44 rats. Rats (n=4 per group) were orally gavaged one time with either 20 mg/kg of Novasoy™, a commercial supplement with a ratio of genistin, daidzin, and glycitin of 1.0:0.5:0.2, or mixtures of synthetic aglycones or glucosides designed to achieve the same isoflavone ratios. The 20 mg/kg mixture of synthetic aglycones contained 11.76 mg genistein, 5.88 mg daidzein, and 2.36 mg glycitein. The 32.4 mg/kg treatment with a mixture of synthetic glucosides was formulated to be equimolar to the mixture of synthetic aglycones and contained 19.09 mg genistin (equal to 11.76 mg genistein), 9.62 mg daidzin (equal to 5.88 mg daidzein), and 3.7 mg glycitin (equal to 2.36 mg glycitein).

Table 39. Isoflavone Absorption and Excretion in Animals

Species	Route	Source	Dose, mg/kg/day[a]	c_{max}[b], nM [µg/L]	t_{max}, hours	Plasma Half-Life, hours	Recovery, %	Reference
Rat (male)	Oral	Genistein	4	**[2250]**	N/A	12.4	N/A	Coldham and Sauer, 2000 *(195)*
Rat (female)				**[601]**	N/A	8.5	N/A	
Rat	Oral	Genistein	20	11,000 **[2970]**	2	8.8	20	King et al., 1996 *(218)* as cited in Whitten and Patisaul, 2001 *(108)*
			45	2200 **[594]**	2	N/A	N/A	Santell et al., 1997 *(211)* as cited in Whitten and Patisaul, 2001 *(108)*
			~40–50	6000–8000 **[1620–2160]**	N/A	3–4	N/A	Chang et al., 2000 *(210)* as cited in Whitten and Patisaul, 2001 *(108)*
Rat	SC	Genistein	500	4.2 µM (24h)	N/A	N/A	N/A	Lamartiniere et al., 1998 *(219)* as cited in Whitten and Patisaul, 2001 *(108)*
Rat	Oral	Soy-derived genistin	20	4900 **[1323]**	2	8.8	18	King et al., 1996 *(218)* as cited in Whitten and Patisaul, 2001 *(108)*
			20	9500 **[2565]**	2	N/A	15	King 1998 *(220)* as cited in as cited in Whitten and Patisaul, 2001 *(108)*
Mouse	Oral	Genistein	45	2600 (free) **[702]**	0.3	4.8	20	Supko and Malspeis 1995 *(221)* as cited in Whitten and Patisaul, 2001 *(108)*
			54–180	4100 (free) **[1107]**	0.05	4.7	21	
			50	1500 (free) **[405]**	0.5	8	11	
	IV	Genistein	52	237,000 **[63,990]**	0.6–1.3	N/A	N/A	
Rat	Oral	Soy-derived daidzein	21	5000 **[1350]**	2	N/A	19	King 1998 *(220)* as cited in Whitten and Patisaul, 2001 *(108)*
Rhesus macaque	Oral	Genistein	7	55 (free+sulfate) **[15]**	N/A	N/A	N/A	Cline et al., 1996 *(222)* as cited in Whitten and Patisaul, 2001 *(108)*
		Daidzein	3	21 (free+sulfate) **[6]**	N/A	N/A	N/A	
Rat	SC	Equol	5	0.4 µM (1 hr)	N/A	N/A	N/A	Gamache and Acworth 1998 *(223)* as cited in Whitten and Patisaul, 2001 *(108)*

Cmax=maximum plasma concentration; tmax=time to cmax, IV=intravenous, SC=subcutaneous, N/A=Not available or not reported.
[a]It is assumed that animals were exposed to the aglycone.
[b]Concentrations expressed in aglycone equivalents. To convert nM to genistein equivalents in µg/L, multiply by 0.27.
Modified and expanded from a review by Whitten and Patisaul *(108)*.

Table 40. Pharmacokinetic Parameters of Plasma Genistein Following Oral and IV Administration of Genistein or Genistein Glucoside (Kwon et al., 2007)

Treatment	*Dose (mg/kg)* [a]	*$AUC_{terminal}$ (ng h/ml)*	*$AUC_{0-\infty}$ (ng h/ml)*	*C_{max} (ng/ml)*	*t_{max} (h)*	*$t_{1/2}$ (h)*	*Absolute Bioavailability (%)* [b]
Oral *(0 – 24 h)*							
Genistein	4	3832 ± 1405	3923 ± 1531	1125 ± 236	0.5 ± 0.1	4.53 ± 1.4	38.58
	20	11,932 ± 2625	12,377 ± 3183	3330 ± 648	0.5 ± 0.1	4.41 ± 1.2	24.34
	40	26,618 ± 5216	31,270 ± 5548	4876 ± 926	2 ± 0.3	5.25 ± 0.9	30.75
IV *(0 – 24 h)*							
Genistein	1	2339 ± 197	2542 ± 203	2943 ± 146		0.20 ± 0.02	
Oral *(0 – 48 h)*							
Genistein-7- glucoside	64 (40 as aglycone equivalents)	48,866 ± 12,450	51,221 ± 12,359	3763 ± 1008	8 ± 1.0	7.47 ± 0.65	48.66
IV *(0 – 48 h)*							
Genistein	1.6 (1 as aglycone equivalents)	2601 ± 390	2631 ± 393	3392 ± 74		0.19 ± 0.01	

IV = intravenous.
[a] Sample sizes were 5 – 6 per treatment group.
[b] Absolute bioavailability (%) = (AUC oral/AUC IV) x (IV dose/oral dose) x 100.
From Kwon et al., 2007 *(226)*.

Table 41. Pharmacokinetic Parameters of Plasma Genistein, Daidzein, and Glycitein in Male and Female Sprague Dawley Rats after a Single Dose (20 mg/kg bw) of Three Sources of Soy Isoflavones (Sepehr et al., 2007)

Treatment[a]	Sex	C_{max} (µmol/L)	t_{max} (h)	$t_{1/2}$ (h)	AUC (µmol/h)/L	Vd/kg (L/kg bw)	Cl/kg (L/h/kg bw)	Absolute Bioavailability (%)[b]
Genistein								
Aglycone	Male	3.5 ± 1.0	6.5 ± 3.0	7.0 ± 1.8	Oral: 19.1 ± 6.7 IV: 34.6 ± 13.3	9.4 ± 4.7	0.9 ± 0.4	29.1 ± 12.4
	Female	2.4 ± 0.3	5.0 ± 3.5	8.3 ± 2.3	Oral: 13.4 ± 1.1 IV: 17.6 ± 3.6	10.3 ± 5.3	1.6 ± 0.4	38.9 ± 5.8
Glucoside	Male	3.9 ± 1.9	5.0 ± 3.5	3.4 ± 2.7	Oral: 30.4 ± 21.1 IV: 31.3 ± 16.2	2.6 ± 1.1	0.6 ± 0.4	57.8 ± 17.4
	Female	2.6 ± 0.9	3.5 ± 3.0	4.7 ± 2.1	Oral: 14.1 ± 4.9 IV: 10.4 ± 2.9	7.3 ± 2.9	1.2 ± 0.4	62.3 ± 8.1
Novasoy	Male	7.7 ± 1.7	4.8 ± 3.8	5.8 ± 2.1	Oral: 49.5 ± 10.4	2.2 ± 0.8	0.3 ± 0.1	62.3 ± 8.1
	Female	3.9 ± 0.7	2.0 ± 0.0	5.8 ± 2.1	Oral: 20.4 ± 2.1	5.9 ± 2.9	0.7 ± 0.2	90.5 ± 5.0
Daidzein								
Aglycone	Male	1.6 ± 0.7	6.5 ± 3.0	9.3 ± 1.5	Oral: 8.5 ± 3.2 IV: 18.9 ± 0.4	14.8 ± 7.3	1.1 ± 0.5	23.0 ± 4.5
	Female	0.6 ± 0.1	5.0 ± 3.5	8.3 ± 2.3	Oral: 3.7 ± 0.4 IV: 7.5 ± 1.4	14.1 ± 3.4	2.8 ± 0.6	25.1 ± 3.6
Glucoside	Male	4.3 ± 1.1	5.0 ± 3.5	7.7 ± 2.4	Oral: 23.3 ± 10.2 IV: 17.0 ± 4.2	3.3 ± 1.9	0.3 ± 0.1	56.9 ± 8.0
	Female	2.2 ± 0.4	3.5 ± 3.0	7.2 ± 0.5	Oral: 13.9 ± 1.8 IV: 10.5 ± 1.6	5.2 ± 0.6	0.5 ± 0.1	54.5 ± 7.8
Novasoy	Male	8.8 ± 1.3	2.0 ± 0.0	8.3 ± 2.7	Oral: 40.3 ± 11.1	1.7 ± 0.5	0.1 ± 0.0	97.7 ± 2.6
	Female	3.2 ± 0.5	2.0 ± 0.0	6.4 ± 2.4	Oral: 18.1 ± 3.2	3.7 ± 1.6	0.4 ± 0.1	88.5 ± 13.1

Table 41. (continued)

Treatment[a]	*Sex*	*C_{max} (µmol/L)*	*t_{max} (h)*	*$t_{1/2}$ (h)*	*AUC (µmol/h)/L*	*Vd/kg (L/kg bw)*	*Cl/kg (L/h/kg bw)*	*Absolute Bioavailability (%)*[b]
Glycitein								
Aglycone	Male	0.05 ± 0.06	8.0 ± 0.0	9.2 ± 2.8	Oral: 0.3 ± 0.1 IV: 2.9 ± 0.7	108.0 ± 71.7	7.7 ± 3.4	4.9 ± 2.1
	Female	0.05 ± 0.06	8.0 ± 0.0	8.7 ± 2.3	Oral: 0.3 ± 0.1 IV: 2.3 ± 0.5	76.1 ± 23.3	6.0 ± 0.9	6.6 ± 1.1
Glucoside	Male	0.5 ± 0.2	8.0 ± 0.0	5.6 ± 0.3	Oral: 1.5 ± 0.4 IV: 1.6 ± 0.2	7.4 ± 4.6	0.9 ± 0.5	57.8 ± 15.8
	Female	0.3 ± 0.1	3.5 ± 3.0	12.8 ± 6.0	Oral: 2.3 ± 0.8 IV: 2.4 ± 1.0	19.6 ± 10.4	1.0 ± 0.1	38.3 ± 4.8
Novasoy	Male	0.5 ± 0.1	3.5 ± 3.0	6.0 ± 2.3	Oral: 2.6 ± 1.0	8.8 ± 3.4	1.1 ± 0.5	68.1 ± 19.9
	Female	0.3 ± 0.2	3.5 ± 3.0	7.8 ± 1.5	Oral: 1.8 ± 0.7	8.0 ± 2.6	1.4 ± 0.4	40.2 ± 16.5

[a] The ratio of genistin, daidzin, and glycitin in the 20 mg/kg diet of Novasoy was 1.0:0.5:0.2. Aglycone and glucoside treatment groups were prepared to achieve this same ratio and have doses that were equimolar with respect to isoflavone content. The 20 mg/kg aglycone diet was composed of 11.76 mg genistein, 5.88 mg daidzein, and 2.36 mg glycitein. The glycoside diet was prepared to take into account differences in molecular weight between the aglycone and glucoside forms and was composed of 19.09 mg genistin (equal to 11.76 mg genistein), 9.62 mg daidzin (equal to 5.88 mg daidzein), and 3.7 mg glycitin (equal to 2.36 mg glycitein).

[b] Absolute bioavailability (%)=(AUC oral/AUC IV) x (IV dose/oral dose) x 100; groups with different superscripts differ significantly

From Sepehr et al., 2007 *(227)*

Animals were also treated by IV injection with mixtures of synthetic aglycones and glucosides in the same proportions used in the oral gavage treatment groups.Absorption was determined by comparing plasma AUC after IV administration with the plasma AUC after oral administration. Absolute bioavailability was also calculated. Bioavailability parameters for Novasoy™ were estimated by comparing AUC_{oral} post-Novasoy administration by AUC_{IV} post-glucoside IV injection. Plasma samples were collected 0-48 hours post-oral gavage treatment or 0-8 hours post-IV dosing. Plasma isoflavones were measured by LC/MS following enzymatic hydrolysis of isoflavone conjugates with a mixture of glucuronidase and sulfatase from *Helix pomatia*. Pharmacokinetic parameters are summarized in **Table 42**. For genistein, bioavailability was significantly higher when administered as the aglycone (35%) compared to when administered as the glucoside genistin (11%). The bioavailability of plasma genistein was lower following ingestion of Novasoy™ when compared to the aglycone (21 versus 35%), but this difference was not statistically different. No statistically significant differences were observed for the bioavailability of plasma daidzein based on treatment: aglycone (34%), glucoside (26%), and Novasoy™ (45%). In contrast to the results for genistein, the bioavailability of glycitein was significantly higher following administration of glycitin (21%) compared to the aglycone (8%). The bioavailability of glycitein following Novasoy™ ingestion (27%) was also significantly higher compared to ingestion of the aglycone, but did not differ from ingestion of the glucoside. Equol was detected by 8 hours post-IV dosing. The authors noted that equol was not detected in a previous study by Sepehr *et al.*, 2007 in younger rats, aged 3 months (described below). The authors conclude that the source of isoflavones has significant effects on the bioavailability of genistein and glycitein.

Jefferson *et al.*, 2009 *(217)* compared serum levels of genistein following administration of 37.5 mg/kg genistein (GEN-37.5) and 60 mg/kg of genistin, which is equivalent to 37.5 mg/kg genistein in aglycone equivalents (GIN-37.5). Female CD-1 [Crl:CD-1 (ICR) BR] mice pups were orally treated with GEN-37.5 or GIN 37.5 on PND 1-5. Trunk blood was collected from 4−6 pups at 0, 0.5, 1, 2, 4, 8, 24, or 48 hours following the last treatment. Genistein aglycone and total genistein was determined by LC-ES/MS/MS before and after enzymatic deconjugation. The pharmacokinetic data are summarized in **Table 43**. The main goal of the study was to assess effects on the female reproductive tract following oral administration of genistein and genistin during neonatal life. Those data are presented in Chapter 3a. The internal exposure following oral administration of genistein aglycone are much lower compared to oral administration of genistin (unadjusted AUC_{agly}=3.6 versus 12.1 µM·h). The authors compared the data for oral administration of genistein or genistin with previously published date by Doerge *et al.*, 2002 *(216)* on CD-1 female mice sc injected with 50 mg/kg genistein (GEN-50). Conclusions related to route of administration are discussed below.

A study by Supko and Malspeis, 1995 *(221)* in mice reported that the systemic bioavailability of genistein was 12% following oral gavage dose of 180 mg/kg genistein. The systemic availability of genistein following an oral treatment with 80.1 mg/kg of genistin was 10.7%.

Route of administration

The UK Committee on Toxicity *(3)* reviewed studies that provided information on absorption and bioavailability of isoflavones. The committee cited a study by Supko and Malspeis, 1995 *(221)* in mice reported that the systemic bioavailability of genistein was about 20% following oral gavage dose of 45 mg/kg and 54.3 mg/kg (differing dosing suspensions) to 20-25 g male Harlan $CD2F_1$ mice (age not given). At higher oral dosages, bioavailability was lower (10%).

Table 42. Pharmacokinetic Parameters of Plasma Genistein, Daidzein, and Glycitein in Aged Male F-344 Rats After a Single Dose (20 mg/kg bw) of Three Sources of Soy Isoflavones (Sepehr et al., 2009)

Treatment[a]	*C_{max} (µmol/L)*	*t_{max} (h)*	*$t_{1/2}$ (h)*	*AUC (µmol/h)/L*	*Vd/kg (L/kg bw)*	*Cl/kg (L/h/kg bw)*	*Absolute Bioavailability (%)*[b]
Genistein							
Aglycone	3.2 ± 0.5	3.5 ± 3.0	5.9 ± 3.0	Oral: 20.0 ± 1.7 IV: 30.7 ± 11.6	8.8 ± 5.1	1.0 ± 0.1	35 ± 9 a
Glucoside	2.1 ± 0.8	6.5 ± 3.0	6.3 ± 0.6	Oral: 12.1 ± 6.7 IV: 43.3 ± 27.1	9.0 ± 3.1	1.0 ± 0.3	11 ± 3 [b]
Novasoy	4.0 ± 1.1	8.0 ± 0.0	5.6 ± 4.4	Oral: 21.3 ± 7.7	4.6 ± 1.2	0.4 ± 0.2	21 ± 7 a,[b]
Daidzein							
Aglycone	1.6 ± 0.4	6.5 ± 3.0	6.2 ± 2.9	Oral: 10.7 ± 1.7 IV: 16.3 ± 4.6	7.0 ± 2.5	0.8 ± 0.2	34 ± 6
Glucoside	2.8 ± 1.1	6.5 ± 3.0	6.9 ± 1.6	Oral: 15.4 ± 7.7 IV: 32.4 ± 18.5	4.3 ± 2.4	0.4 ± 0.2	26 ± 5
Novasoy	5.2 ± 1.7	6.5 ± 3.0		Oral: 32.4 ± 11.8			45 ± 18
Glycitein							
Aglycone	0.1 ± 0.0	8.0 ± 0.0	7.3 ± 5.5	Oral: 0.7 ± 0.1 IV: 4.2 ± 0.9	67.7 ± 8.9	1.4 ± 1.6	8 ± 3 a
Glucoside	0.4 ± 0.1	8.0 ± 0.0	9.3 ± 1.7	Oral: 1.8 ± 0.7 IV: 3.5 ± 0.2	11.0 ± 3.5	0.8 ± 0.3	21 ± 10 [b]
Novasoy	0.4 ± 0.1	6.5 ± 3.0	8.2 ± 0.6	Oral: 2.2 ± 0.8	9.23 ± 5.1	0.8 ± 0.4	27 ± 13 [b]

[a] The ratio of genistin, daidzin, and glycitin in the 20 mg/kg diet of Novasoy was 1.0:0.5:0.2. Aglycone and glucoside treatment groups were prepared to achieve this same ratio and have doses that were equimolar with respect to isoflavone content. The 20 mg/kg aglycone diet was composed of 11.76 mg genistein, 5.88 mg daidzein, and 2.36 mg glycitein. The glycoside diet was prepared to take into account differences in molecular weight between the aglycone and glucoside forms and was composed of 19.09 mg genistin (equal to 11.76 mg genistein), 9.62 mg daidzin (equal to 5.88 mg daidzein), and 3.7 mg glycitin (equal to 2.36 mg glycitein).

[b] Absolute bioavailability (%)=(AUC oral/AUC IV) x (IV dose/oral dose) x 100.
Groups with different superscripts differ significantly.

From Sepehr et al., 2009 *(228)*.

Table 43. Comparison of Serum Circulating Levels of Genistein Following Oral Administration of Genistein or Genistin or SC Injection with Genistein (Jefferson et al., 2009)

Treatment (mg/kg)	AUC_{tot}			AUC_{agly}		
	Unadjusted (μM·h)	Dose Corrected (AUC_{tot}/dose)	% SC	Unadjusted (μM·h)	Dose Corrected (AUC_{agly}/dose)	% SC
GEN-50 (SC)	147	2.9	100	33	0.66	100
GIN-37.5 (Oral)	90.4	2.4	83	12.1	0.32	48
GEN-37.5 (Oral)	12.8	0.34	12	3.6	0.10	15

SC = subcutaneous
From Jefferson et al., 2009 *(217.)*

The authors stated that dissolution problems were likely the cause of poorer bioavailability. Systemic bioavailability following an ip injection of 185 mg/kg bw was reported as 58%. On average, the plasma levels following ip injection with 185 mg/kg genistein were about 5 times higher than levels observed following the oral dose of 180 mg/kg oral. **[The expert panel notes that the validity of the plasma data following the oral dosing is uncertain based on the authors concern about dissolution. In addition, the relevance of the data in mice that are presumed to be mature based on the reported weights (20-25grams) to the question of isoflavones in immature rodents and/or humans is limited.]**

A study of genistein bioavailability in adult (8-week-old) ovariectomized Sprague Dawley rats *(214)* demonstrated that genistein aglycone represented 12–23% of total genistein levels with dietary exposure and 44–48% of total genistein levels at the 2 highest sc doses. The study authors noted the higher levels of free genistein with sc compared to dietary dosing.

[The Expert Panel noted that the differences in bioavailability with oral versus parenteral exposure noted in both studies above is consistent with first pass metabolism either within the gut lumen, gut wall and/or liver. The values presented in the latter study are consistent with the body of data presented by others, although it is noted that studies were conducted using different methods. In general, serum genistein aglycone levels in adult rats were observed at ~1–20% following oral exposure and ~40–50% following sc exposure. However, although these data are interesting, they do not inform the question of first pass effects in immature animals or humans.]

The study by Jefferson *et al.*, 2009 *(217)* described above compared serum levels of genistein following administration of 37.5 mg/kg genistein (GEN-37.5) and 60 mg/kg of genistin, which is equivalent to 37.5 mg/kg genistein in aglycone equivalents (GIN-37.5) with previously published data by Doerge *et al.*, 2002 *(216)* on CD-1 female mice sc injected with 50 mg/kg genistein (GEN-50) (**Table 43**). As noted above, the internal exposure following oral administration of genistein aglycone are much lower compared to oral administration of genistin (unadjusted AUC_{agly}=3.6 versus 12.1 μM·h). With respect to differences based on route of administration, the dose-adjusted AUC_{tot} were similar for genistein administered by sc injection and oral genistin (2.9 versus 2.4 AUC_{tot}/dose). The dose-adjusted AUC_{agly} were approximately 2-fold higher for genistein administered by sc injection and oral genistin (0.66 versus 0.32 AUC_{agly}/dose), which the authors attribute to differences in percent aglycone AUC between genistein administered by sc injection (22% of total AUC) and

orally administered genistin (13% of total AUC) due to the bypass of Phase II conjugation in the gut following injection. The authors conclude that oral genistin is quickly hydrolyzed and absorbed in neonates.

Doerge *et al.*, 2002*(216)*, supported by the NCTR/FDA, NIEHS, and NTP, examined the pharmacokinetics of genistein administered by sc injection to neonatal mice. Male and female CD-1 mice were injected on PND 1–5 with genistein **[purity not given]** in corn oil at 0 or 50 mg/kg bw/day. The mice (n=3–8/sex/time period) were killed on PND 5 at time intervals between 0.5 and 24 hours following exposure, and blood was collected for a determination of toxicokinetic parameters. Levels of conjugated and unconjugated isoflavones were measured in serum using LC- electrospray MS. Toxicokinetic parameters are summarized in **Table 44**, and serum levels of total and aglycone genistein are reported in **Table 38**.

Table 44. Toxicokinetic Parameters in Neonatal Mice Given Genisteinby SC Injection (Doerge et al., 2002)

Genistein Form	*Sex*	*Elimination Half-Life, hour*	*AUC, nM-hour* [µg-hour/L]	*V_d, (L/kg)*	*C_{max}, nM* [µg/L]
Aglycone	Female	12	33 **[9]**	99	2300 **[621]**
	Male	16	38 **[10]**	112	1400 **[378]**
Conjugate	Female	19	114 **[31]**	Not reported	5000 **[1350]**
	Male	16	121 **[33]**	Not reported	3000 **[810]**

SC = subcutaneous
The genistein dose was 50 mg/kg bw/day for 5 days.
From Doerge et al., 2002 *(216)*..

The maximum serum concentration was reached in both sexes at 0.5 hours, the earliest sampling time point. In males and females, ~31% of genistein was present in aglycone form. **[Based on Figures 2 and 3 of the study report, it appears that 31% aglycone was the mean value throughout the time period; values ranged from ~20 to 40%.]** In a comparison with data generated in other studies, the percentage of aglycone was higher in neonatal mice than in adult rats (1–3%) and mice (6–16%) fed genistein in aglycone form. Compared to aglycone levels in fetuses or neonates of rats orally dosed with genistein during the gestation or lactation period, neonatal aglycone levels in this study were similar or lower than values reported in 1 study (31–53%) *(208)* but higher than values reported in a second study (14–19%) *(209)*. The authors suggested that in addition to exposure route differences, interspecies and developmental factors could be responsible for variations in aglycone levels reported in different studies. The study authors concluded that metabolic differences between perinatal and adult animals have a greater impact on aglycone levels than route of administration.

Because these two studies were performed by the same authors with a similar dosing design and both address the *neonatal* CD-1 mouse model, the results are deemed highly relevant and of utility. However, it is important to note that a comparison of the C_{max} data from Jefferson *et al.* 2009*(217)* and Doerge *et al.*,2002 *(216)* are puzzling. In contrast to the AUC data, the C_{max} values were lower following subcutaneous exposure in Doerge *et al.*, 2002 *(216)* compared to those following oral exposure in Jefferson *et al.* 2009 *(217)*. Nevertheless, because no AUC data are available for genistein in infants consuming soy formula (only single concentrations presumably at steady state) for purposes of comparing the neonatal mouse data to the human infant data, the C_{max} data are relevant. As such,

the serum C_{max} values observed with these dosing schema range from 4.4 to 19 µM total genistein and 1.4 to 5 µM unconjugated aglycone, within the range of values observed for human infants.

Life stage

[Comparisons of serum aglycone levels in adult and fetal or neonatal rodents of the same study can be made from the rat data presented in Table 38. A sc dosing study conducted in rats demonstrated similar percentages of serum aglycone (35–46%) at PND 21, 50, or 100. One study with gavage exposure demonstrated higher aglycone percentages in fetuses (27–34%) than dams (5–18%) on GD 20 or 21 *(208)*. A dietary study in which dams were fed 25 or 250 ppm genistein did not consistently demonstrate higher percentages of aglycone in dams (1.7–23%) compared to pups on PND 7 (14–19%) or PND 21 (6.6–33%) *(209)*. In an evaluation of all the data, percentages of free genistein following oral exposure of adult rats are usually below 10% but sometimes attain levels of ~20%. Percentages of aglycone following direct and/or indirect oral exposure to genistein in rat pups ≤21 days old were reported at 1–33%.]

A study by Cotroneo *et al.*, 2001*(215)* demonstrated that sc injection of rats with 500 mg/kg bw genistein on PND 21, 50, or 100 resulted in blood genistein levels that were ~2 orders of magnitude higher on PND 21 versus PND 50 or 100 (Table 38). **[The Expert Panel noted that the higher blood genistein levels on PND 21 indicate reduced clearance in immature rats].**

2.1.2.2 Distribution

Distribution in adults

Chang *et al.*, 2000 reported total and aglycone genistein levels in a number of reproductive and non-reproductive tissues following dietary administration of genistein aglycone. The fraction present as aglycone ranged from 11% (testes) to 100% (brain and uterus) (see below). Higher free genistein levels in rat tissues than rat blood were demonstrated by McClain *et al.*, 2004*(229)*. Male and female rats were fed diets providing genistein doses of 0.5–500 mg/kg bw/day for 4 weeks or 5–500 mg/kg bw/day for 13, 26, or 52 weeks. In plasma, free genistein represented small amounts of the total genistein value **[most often ≤3%; one sample had a mean value of 22%]**. Percentages of free genistein were higher in liver and kidney than plasma. The study authors could not provide an explanation for the higher levels of free versus total genistein levels in some liver samples. Total blood genistein levels in males ranged from 504 to 1896 nM **[136 to 512 µg/L]** at 5 mg/kg bw/day, 3871 to 16,227 nM **[1046 to 4385 µg/L]** at 50 mg/kg bw/day, and 22,560 to 52,319 nM **[6097 to 14,139 µg/L]** at 500 mg/kg bw/day. The equivalent blood concentration in females rats at each dose level were 169–2053 nM **[46–555 µg/L]**, 1947–6192 nM **[526–1673 µg/L]**, and 22,250–90,686 nM **[6013–24,507 µg/L]**.

As discussed earlier, Coldham and Sauer 2000 *(195)* reported data on tissue distribution, mass balance, and plasma pharmacokinetics of rats gavaged with 4 mg/kg bw ^{14}C-genistein. The concentration of [^{14}C]genistein was significantly (P<0.002) higher in liver from females than males and in reproductive (vagina, uterus, ovary, and prostate) compared with other peripheral organs (Table 45). This same trend was present for other organs such as brain, fat, thymus, spleen, skeletal muscle, and bone. The V_d was reported at 1.27–1.47 L. **[This finding suggests that most of the circulating radioactivity was not genistein but the glucuronide. Plasma protein binding ranged from 77.3 to 97.7%, with males exhibiting much higher binding than females. It is possible that this gender difference was due to much higher levels of 17β-estradiol in females, which would displace genistein from**

Table 45. Average Tissue Distribution in Male and Female Rats Following Oral Dosing with 4 mg/kg [^{14}C] Genistein (Coldham and Sauer, 2000)

Tissue Type	Tissue	Male (ng/g tissue)			Female (ng/g tissue)		
		2h	7h	24h	2h	7h	24h
Gut	Stomach	8,102	4,680	498	7,394	3,205	339
	Small intestine	18,458	11,894	1,088	25,867	18,864	2,714
	Cecum	478	17,397	1,920	288	1,920	2,146
Excretory Organs	Liver	980	2,666	1,062	1,062	236	314
	Kidney	686	515	57	269	399	71
Respiratory Organs	Heart	121	119	35	133	155	33
	Lung	300	304	74	399	419	82
	Plasma	785	779	116	836	839	163
	Red blood cells	151	155	21	95	114	22
Peripheral Organs	Brain	69	22	10	28	17	4
	Fat (abdominal)	44	37	12	35	51	11
	Thymus	95	57	45	63	89	26
	Spleen	84	63	50	85	83	21
	Skeletal muscle	13	17	6	16	15	4
	Bone	46	46	37	41	42	13
Reproductive Organs	Testis/ovary	117	96	23	250	259	57
	Prostate/uterus	278	454	250	253	293	77
	Vagina	–	–	–	322	417	289

n=4/sex at each time point.
From Coldham and Sauer, 2000 *(195)*.

protein binding sites. The shorter half-life in females than in males is compatible with a rough correlation between protein binding and half-life of drugs.]

Goelzer *et al.*, 2001 *(230)* characterized the tissue distribution of 3-(^{14}C)-genistein administered as a single oral gavage dose of 5 or 50 mg/kg to male and female rats. For tissue distribution, radioactivity was determined for an extensive tissue list by quantitative whole body autoradiography at 3, 6, 12, 24 and 72 hours (n=15/sex/dose level). In a separate set of biliary cannulated animals (n=3/sex/dose level), bile was collected at 2, 4, 6, 12, 24 and 48 hours and replaced with control donor bile; excreta were collected at 24 and 48 hours and the radioactive content was determined. Most tissues had been exposed to radioactivity at the first time point, 3 h following dose administration (see **Table 46** and **Table 47**). Generally, maximum concentrations of radioactivity were achieved at 3 h post dose (first sampling time) in male animals but were not achieved until 6 h at the low dose level and at 12 h at the high dose level in female animals, suggesting a sex difference in the routes and rates of absorption or metabolism. Considering that at 12 h in plasma the radioactivity is almost entirely reflecting metabolites the radioactivity in the tissues at later time points could also be caused by metabolites. The concentrations of radioactivity in the tissues exhibited a linear relationship to the dose level. With the exception of a few tissues, a 10-fold increase in dose gave between an approximately 4 to 16 fold increase in the C_{max} values indicating that the routes of absorption had not been saturated. Radioactivity was essentially cleared from the tissues within 24 h of dose administration and there was no evidence of accumulation in any tissue.

Table 46. Mean Concentration of Radioactivity Following Oral Administration of [^{14}C] Genistein to Rats at a Dose Level of 5 mg/kg (Goelzer et al., 2001)

Tissue Type	*Tissue*	*Males*				*Females*			
		3 h	*6h*	*12h*	*24h*	*3 h*	*6h*	*12h*	*24h*
Vascular/ Lymphatic	Aorta	0.903	0.403	0.321	BLQ	0.116	0.499	0.193	BLQ
	Blood	1.141	0.549	0.434	BLQ	0.098	0.523	0.366	BLQ
	Lymph Nodes	0.444	0.510	0.147	BLQ	BLQ	0.236	0.170	BLQ
Metabolic/ Excretory	Kidney Cortex	1.717	1.506	0.998	0.102	0.419*	2.109	0.736	0.073
	Kidney Medulla	1.587	1.515	1.291	0.066	0.279*	1.429	0.610	0.060
	Liver	2.011	0.835	0.447	BLQ	0.288	0.768	0.258	BLQ
CNS	Brain/Spinal Cord	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ
	Pineal Body	0.285	0.187	0.150	BLQ	0.026	0.080	0.120	BLQ
Endocrine	Adrenal	0.639	0.250	0.240	BLQ	0.105	0.411	0.217	BLQ
	Pituitary	0.251	0.129	0.086	BLQ	BLQ	0.122	0.049	BLQ
	Thymus	0.194	0.135	0.095	BLQ	BLQ	0.130	0.053	BLQ
	Thyroid	0.378	0.155	0.132	BLQ	BLQ	0.194	0.095	BLQ
Secretory	Harderian Gland	0.246	0.121	0.104	BLQ	BLQ	0.136	0.070	BLQ
	Lacrimal Gland	0.447	0.175	0.143	BLQ	BLQ	0.224	0.149	BLQ
	Salivary Glands	0.297	0.201	0.110	BLQ	BLQ	0.124	0.095	BLQ
Fatty	Brown Fat	0.608	0.257	0.154	BLQ	0.056	0.209	0.161	BLQ
	White Fat	0.133	0.077	0.068	BLQ	BLQ	0.263*	0.028	BLQ
Muscular	Muscle	0.115	0.094	0.060	BLQ	BLQ	0.076	BLQ	BLQ
	Myocardium	0.480	0.227	0.166	BLQ	BLQ	0.253	0.137	BLQ
	Tongue	0.539	0.235	0.180	BLQ	BLQ	0.243	0.149	BLQ

Table 46 (continued)

Tissue Type	Tissue	Males				Females			
		3 h	6h	12h	24h	3 h	6h	12h	24h
Gonads	Bulbo–urethral	0.602	0.321	1.022	BLQ	–	–	–	–
	Epididymis	0.201	0.166	0.076	BLQ	–	–	–	–
	Preputial	0.444	0.152	0.147	BLQ	–	–	–	–
	Prostate	0.307	1.243*	0.259	BLQ	–	–	–	–
	Seminal Vesicles	0.058	0.177	0.101	BLQ	–	–	–	–
	Testes	0.358	0.189	0.124	BLQ	–	–	–	–
	Clitoris	–	–	–	–	0.070	0.172	0.149	0.029
	Ovary	–	–	–	–	0.106*	0.413*	0.272	BLQ
	Uterus	–	–	–	–	0.104*	0.618*	0.233*	BLQ
Unclassified	Bone Marrow	0.203	0.116	0.119	BLQ	0.033	0.112	0.032	BLQ
	Epimysium	0.542	0.261	0.183	BLQ	0.049	0.201	0.103	BLQ
	Lung	0.887	0.433	0.285	BLQ	0.055	0.493	0.266	BLQ
	Nasal Mucosa	0.192	0.145	0.095	BLQ	BLQ	0.047	0.046	BLQ
	Pancreas	0.409	0.547	0.173	BLQ	0.352*	0.625*	0.118	BLQ
	Skin	0.417	0.258	0.161	0.052	0.028	0.174	0.115	0.062
	Spleen	0.543*	0.141	0.103	BLQ	0.116*	0.209*	0.055	BLQ
	Tooth Pulp	0.779	0.293	0.283	BLQ	0.029	0.254	0.192	BLQ
	Uveal Tract	0.372	0.187	0.163	BLQ	0.034	0.185	0.101	BLQ
GI Mucosa	Stomach	0.743*	0.263	0.177	BLQ	0.504*	0.668	0.091	BLQ
	Small Intestine	0.526*	3.097*	1.067*	BLQ	0.240*	2.801*	0.982	BLQ
	Cecum	1.130*	10.84*	1.566	BLQ	2.341*	2.431*	0.908	BLQ
	Large Intestine	0.378*	2.444*	1.043	BLQ	0.425*	1.374*	0.486	BLQ
	Rectum	0.376	0.242*	0.973*	BLQ	0.063	0.477	0.617*	BLQ

BLQ=Below the limit of quantification.
*Measurement affected by Flaring.
From Goelzer et al., 2001 *(230)*.

Table 47. Mean Concentration of Radioactivity Following Oral Administration of [^{14}C] Genistein to Rats at a Dose Level of 50 mg/kg (Goelzer et al., 2001)

Tissue Type	*Tissue*	*Males*				*Females*			
		3 h	*6h*	*12h*	*24h*	*3 h*	*6h*	*12h*	*24h*
Vascular/ Lymphatic	Aorta	6.022	4.636	5.089	BLQ	2.282	2.169	4.640	BLQ
	Blood	7.427	5.013	6.258	BLQ	1.605	3.683	7.364	0.489
	Lymph Nodes	2.400	1.498	2.160	BLQ	0.330	1.049	2.613	BLQ
Metabolic/ Excretory	Kidney Cortex	13.63	13.40	15.40	0.736	5.622	8.089	15.20	1.428
	Kidney Medulla	10.59	9.342	14.80	BLQ	3.471	8.071	14.63	1.804
	Liver	13.26	6.947	6.831	BLQ	7.782	5.196	5.924	0.480
CNS	Brain	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ
	Spinal Cord	BLQ	BLQ	BLQ	BLQ	0.222	BLQ	BLQ	BLQ
	Pineal Body	1.819	1.406	1.532	BLQ	0.236	0.884	1.680	BLQ
Endocrine	Adrenal	4.711	2.798	3.271	BLQ	3.240	0.648	3.658	0.382
	Pituitary	1.405	0.933	1.186	BLQ	0.488	0.533	1.459	BLQ
	Thymus	1.444	0.812	1.182	BLQ	BLQ	0.556	1.248	BLQ
	Thyroid	1.864	1.656	1.973	BLQ	0.498	1.074	1.350	BLQ
Secretory	Harderian Gland	1.414	1.107	1.324	BLQ	0.224	1.153	1.534	BLQ
	Lacrimal Gland	2.289	1.688	1.647	BLQ	0.579	0.787	2.204	0.259
	Salivary Glands	1.752	1.176	1.448	BLQ	0.238	0.955	1.907	BLQ
Fatty	Brown Fat	2.538	1.744	2.169	BLQ	0.746	1.720	2.258	0.288
	White Fat	0.276	0.695	0.956	BLQ	BLQ	0.515	1.447	0.229
Muscular	Muscle	0.792	0.549	0.953	BLQ	BLQ	BLQ	0.793	BLQ
	Myocardium	2.862	2.071	2.542	BLQ	0.355	1.529	2.871	0.215
	Tongue	1.613	1.374	2.311	BLQ	0.322	1.090	2.493	BLQ

Table 47 (continued)

Tissue Type	Tissue	Males				Females			
		3 h	6h	12h	24h	3 h	6h	12h	24h
Gonads	Bulbo–urethral	12.68	9.689	3.533	BLQ	–	–	–	–
	Epididymis	1.531	1.249	1.907	BLQ	–	–	–	–
	Preputial	1.767	1.262	1.956	BLQ	–	–	–	–
	Prostate	1.684	1.315	1.596	BLQ	–	–	–	–
	Seminal Vesicles	0.276	0.684	0.558	BLQ	–	–	–	–
	Testes	1.549	1.313	1.533	BLQ	–	–	–	–
	Clitoris	–	–	–	–	1.305	2.942	2.800	BLQ
	Ovary	–	–	–	–	2.269	4.756	7.156	1.219
	Uterus	–	–	–	–	1.956	4.796	5.147	BLQ
Unclassified	Bone Marrow	1.347	0.972	1.263	BLQ	BLQ	0.550	1.428	BLQ
	Epimysium	2.884	2.432	2.258	BLQ	0.550	1.413	2.387	BLQ
	Lung	5.702	4.476	4.307	BLQ	1.302	2.916	5.920	0.396
	Nasal Mucosa	1.475	0.847	1.526	BLQ	0.681	1.162	1.095	BLQ
	Pancreas	6.902	4.671	3.013	BLQ	1.586	1.507	8.580	BLQ
	Skin	2.649	2.043	2.178	BLQ	1.082	0.982	2.222	0.554
	Spleen	2.609	1.493	1.662	BLQ	2.812	1.253	2.268	BLQ
	Tooth Pulp	5.527	1.927	4.631	BLQ	1.016	2.520	4.951	BLQ
	Uveal Tract	2.009	1.982	2.511	BLQ	0.813	1.476	1.853	0.281
GI Mucosa	Stomach	5.538	4.000	2.404	BLQ	9.169	1.234	2.236	BLQ
	Small Intestine	6.591	15.46	19.36	BLQ	12.87	20.58	19.70	BLQ
	Cecum	4.476	54.78	10.49	0.892	7.916	51.57	18.38	BLQ
	Large Intestine	1.600	0.807	6.280	BLQ	4.257	4.616	22.91	BLQ
	Rectum	2.027	1.947	10.03	BLQ	0.967	1.336	3.773	BLQ

BLQ = Below the limit of quantification.
From Goelzer et al., 2001 *(230)*.

Table 48. Phytoestrogen Levels in Brains of Male Rats Fed a Phytoestrogen-Containing Diet (Lund et al., 2001)

Brain Region	Isoflavone Levels, µg/kg [nmol/g][a]			
	Daidzein	Genistein	Equol	Total
Frontal cortex	271.0 ± 29.8 **[1.07 ± 0.12]**	295.5 ± 35.4 **[1.09 ± 0.13]**	705.3 ± 63.4 **[2.91 ± 0.26]**	1272 ± 129 **[5.07 ± 0.51]**
Hippocampus	3.4 ± 0.4 **[0.013 ± 0.0016]**	3.1 ± 0.3 **[0.011 ± 0.0011]**	28.5 ± 3.4 **[0.12 ± 0.014]**	35.0 ± 4.1 **[0.14 ± 0.017]**
Amygdala	5.5 ± 0.6 **[0.022 ± 0.0024]**	9.9 ± 1.1 **[0.037 ± 0.0041]**	57.5 ± 5.2 **[0.24 ± 0.021]**	72.9 ± 6.9 **[0.30 ± 0.028]**
Cerebellum	58.8 ± 29.8 **[0.23 ± 0.12]**	126.8 ± 29.8 **[0.47 ± 0.12]**	33.4 ± 29.8 **[0.14 ± 0.12]**	219.0 ± 29.8[b] **[0.84 ± 0.36]**

[a] Data presented as mean ± SEM.
[b] **[SEMs reported by study authors; there appears to be an error because all were the same.]**
From Lund et al., 2001 *(231)*.

Isoflavones were detected in brains of adult male rats fed a soy-based diet containing 600 ppm phytoestrogens *(231; 232)*. As noted in **Table 48**, total isoflavones were greatest in frontal cortex > cerebellum > amygdala > hippocampus. In hippocampus, isoflavone levels were similar to those of rats fed a phytoestrogen-free diet. In the frontal cortex and amygdala, the concentration of equol was > genistein > daidzein. In the cerebellum, genistein > daidzein > equol. The study authors stated that cerebellum and frontal cortex contained an abundance of estrogen receptor (ER) β. Levels of phytoestrogens in the medial basal hypothalamic and preoptic areas were reported at 4.4 ng/g **[0.017 nmol/g]** daidzein, 3.5 ng/g **[0.013 nmol/g]** genistein, and 126 ng/g **[0.52 nmol/g]** equol *(232)*. Levels of genistein and equol were significantly higher than in rats fed a phytoestrogen-free diet.

Chen *et al.*, 2006 *(233)* compared the steady state concentrations of genistein in the plasma, liver, and skeletal muscle of 6 adult (1-year old) and 4 old age (2-years old) Sprague-Dawley rats following a 5-week treatment with equivalent feed doses of 154 or 308 ppm genistein. Animals fasted for 12 hours prior to sample collection. At both doses, the concentrations of genistein in the old-age animals were significantly lower compared to the younger adult rats in plasma. For liver and skeletal muscle, genistein levels were either similar or altered in the old-age rats, depending on dietary concentration (**Table 49**). There was a linear relationship between diet concentration and genistein concentration in the plasma, liver and muscle for adult but not old-age animals (r^2=0.76, 0.86, and 0.64). The authors also noted that in 1-year old animals fed a diet of 62 ppm genistein, the plasma concentrations

Table 49. Mean Genistein Levels in Plasma, Liver, and Skeletal Muscle of Adult and Old Rats Fed Genistein in the Diet for 5 Weeks (Chen et al., 2006)

Genistein in Diet	Plasma, µmol/L		Liver, nmol/g		Skeletal Muscle, nmol/g	
	Adult	Old	Adult	Old	Adult	Old
154 ppm	0.74	0.40*	0.40	0.29	0.11	0.32*
308 ppm	1.12	0.53*	0.74	0.37*	0.35	0.33

*Means are significantly different between adult and old-age animals.
From Chen et al., 2006 *(233)*.

of genistein were not significantly different from control animals whereas levels in the liver and skeletal muscle were significantly higher compared to control animals, suggesting a longer half-life of genistein in these tissues compared to blood.

Penza *et al.*, 2007 *(234)* reported that fasting induces genistein mobilization in mice. In this study, mice were treated with a loading dose of 50 mg/kg/day of genistein for three days and then fasted for 48 hours, starting on day 15 from loading. Serum concentrations of genistein were measured at baseline, pre-fasting (day 15 from loading), and post-fasting (day 17 from loading). Serum genistein was significantly higher following the 48-hour fasting period compared to pre-fasting measurements (99 versus 163 nM).

Distribution to the fetus and lactational transfer following maternal ingestion

More information on the distribution of isoflavones during pregnancy and lactation is available for genistein compared to the other isoflavones. The studies described below show fetal transfer for genistein. Similar to genistein, Janning *et al.*, 2001 *(235)* reported that the placenta does not represent a barrier to daidzein based on a toxicokinetic study conducted in pregnant DA/HAN rats.

Weber *et al.*, 2001 *(236)* assessed placental and lactational transfer of isoflavones as part of a developmental toxicity study that is discussed in detail in Chapter 3. Throughout pregnancy and lactation, 10 Sprague Dawley rats received a commercially available high-phytoestrogen diet. The diet contained phytoestrogens 603 µg/g feed consisting mainly of daidzin, genistin, and glycitin. The diet was soy-based, according to Lephart *et al.*, 2002 *(237)*. At gestation day (GD) 20.5 **[day of plug not specified]** and on postnatal day (PND) 3.5 **[day of birth not specified],** blood was collected and pooled from dams and offspring (n=3–5 litters at each collection period) for measurement of total plasma genistein, daidzein, and equol levels by GC-MS. Blood levels of phytoestrogens in adult male rats fed the same diets were also examined. As noted from the study results listed in **Table 50**, gestational and lactational transfer of isoflavones was demonstrated. GD 20.5 dams were noted to have lower phytoestrogen plasma levels than male rats. The study authors proposed that changes in phytoestrogen metabolism or increased circulatory volume in late pregnancy were possible reasons for the lower plasma phytoestrogen levels in GD 20.5 dams.

Table 50. Isoflavone Levels in Plasma of Rat Dams and Offspring Following Maternal Ingestion of a Diet Containing 603 µg/g Feed of Phytoestrogens (Weber et al., 2001)

Age	*Plasma Aglycone Equivalent Levels, µg/L* [nM]		
	Genistein	*Daidzein*	*Equol*
Fetus, GD 20.5	106.39 **[394]**	67.53 **[266]**	51.58 **[213]**
Dam, GD 20.5	232.31 **[860]**	193.80 **[762]**	578.12 **[2387]**
Pup, PND 3.5	234.85 **[869]**	341.13 **[1342]**	161.85 **[668]**
Dam, PND 3.5	441.22 **[1633]**	339.46 **[1335]**	906.22 **[3742]**
Adult male	420.95 **[1558]**	390.27 **[1535]**	932.37 **[3850]**

From Weber et al., 2001 *(236)*.

Brown and Setchell *(238)* measured serum levels of isoflavones in adult mice and rats and in fetuses and pups of rats fed various rodent diets. In adult FVB mice fed 1 of 3 soy-based diets (Purina 5008 or 5010 or NIH-07), serum levels of total genistein and daidzein **[≤100 ng/mL each; ≤370 nM genistein, ≤393 nM daidzein]** were about an order of magnitude lower than serum total equol

levels **[~ 1300–2300 ng/mL]**. Similar results were observed in Sprague Dawley rats fed a soy-based diet (Purina 5001), with serum levels of total equol **[~2100 ng/mL]** greatly exceeding those of total genistein and daidzein **[~ 100 ng/mL each; ≤370 nM genistein, ≤393 nM daidzein]**. Urine levels of genistein and daidzein glucuronide were about 25% of equol glucuronide levels in mice and 50% of equol glucuronide levels in rats. Results of serum analysis in rat pups of dams fed Purina 5001 are presented in **Table 51**. The total concentration of isoflavones in the Purina 5001 diet was 810 µg/g. The concentrations of the major individual isoflavones were: genistein (4 µg/g), genistin (318µg/g), daidzein (59 µg/g), daidzin (262 µg/g), and glycitin (53 µg/g). According to the study authors, serum isoflavones in newborn pups prior to nursing represent maternal-fetal transfer during gestation. The authors stated that pups do not start eating feed until 15–16 days of age, and therefore serum levels of isoflavones in pups before that time point were also of maternal origin. Equol levels were very high at birth and rapidly declined during the postnatal period. Stomach contents of newborn rats, presumed to be swallowed amniotic fluid, also contained high levels of isoflavones consisting of 44% genistein derivatives, 37% equol derivatives, and 19% daidzein derivatives.

Table 51. Serum Isoflavone Levels in Rodent Offspring fed a Soy-Based Diet (Brown and Setchell, 2001)

Pup Age	*Aglycone Equivalents, ng/mL*[a] **[nM]**		
	Genistein	*Daidzein*	*Equol*
Newborn[b]	100 **[370]**	50 **[197]**	550 **[2271]**
6-day-old	90 **[333]**	50 **[197]**	80 **[330]**
12-day-old	110 **[407]**	25 **[98]**	70 **[289]**
16-day-old	10 **[37]**	5 **[20]**	60 **[248]**

[a] Values estimated from a graph by CERHR.
[b] Measured in pups prior to nursing.
From Brown and Setchell 2001 *(238)*.

Doerge *et al.*, 2001*(208)* evaluated the appearance of maternally administered genistein (>99% purity) in Sprague Dawley rat pups evaluated shortly after birth. Pregnant animals were exposed either in the diet or by gavage. The basal diet was a soy- and alfalfa-free diet (5K96) in which genistein and daidzein levels were determined using HPLC-MS analysis (after hydrolysis of glucoside conjugates) to be 0.54 µg/g feed (genistein) and 0.48 µg/g feed (daidzein). Animals treated with dietary genistein were given feed with genistein aglycone 500 µg/g feed **[500 ppm; 0.05%].** Based on feed consumption of 30 g/day and 300 g rat weight, the authors estimated daily genistein doses of 0.05 and 50 mg/kg bw with control and genistein-supplemented diets, respectively **[neither feed intake nor body weight were reported]**. Genistein was measured in excess pups that were born in a multigeneration study. **[The duration of treatment was not specified in the current paper, but in a preliminary study by these authors *(239)*, genistein-supplemented feed was given from the day a vaginal plug was detected (GD 0).]** The pups were killed at the time of litter standardization on PND 1 or 2. Trunk blood was collected by decapitation. Eight dams on the genistein-treated diet contributed 18 individual pups plus an additional 2 samples that were pooled from 2 or more pups in the same litter. Total serum genistein levels in pups were measured at a mean ± SD of 176 ± 307 nM **[corresponding to 48 ± 83 µg/L genistein aglycone equivalents]**; genistein aglycone was measured at 47 nM **[13 µg/L]**, or 53% of the total genistein. **[CERHR calculated that aglycone represents 27% of total genistein. The Expert Panel noted**

that the large SDs suggest a skewed distribution for which the mean may not be the best estimate of central tendency. The paper noted that the mean ± SD serum concentration from the 8 litters (presumably unpooled fetuses) born to dams given genistein-supplemented feed was 216 ± 282 nM (genistein agylcone equivalent 58 ± 76 µg/L) with a range of 46–955 nM (corresponding to genistein aglycone 12–258 µg/L).] Four pups were analyzed from 2 litters exposed to the control diet, giving a mean ± SD total genistein level of 3 ± 1 nM **[genistein aglycone equivalent 0.8 ± 0.3 µg/L]**.

In a separate experiment, female Sprague Dawley rats were maintained on the soy- and alfalfa-free diet for life. Animals were mated **[age not specified]**, and 20 or 21 days after a vaginal plug, a single gavage dose of genistein was given. Dose levels were 20, 34, and 75 mg/kg bw **[n=1 pregnant animal per dose]**. Pregnant rats were killed 2 hours after the gavage treatment, and fetuses were surgically removed. Trunk blood was collected by decapitating fetuses, and maternal blood was collected by cardiac puncture. **[It is not indicated whether fetal blood was pooled within litters or analyzed separately for each fetus. Adult concentrations are presented as single values without SD, and offspring values are presented as mean ± SD, suggesting that single dams were used for each dose group and that fetuses were analyzed individually. A subsequent comment in the Results section raises the possibility that fetal sera were pooled for analysis, which would make inexplicable the use of mean and SD.]** Maternal and fetal brains were frozen for later analysis of tissue genistein. Serum total and aglycone genistein levels are summarized in **Table 38**. Brain genistein levels are shown in **Table 52**.

Table 52. Brain Genistein Concentration After a Single Maternal Gavage Dose of Genistein on GD 20 or 21 (Doerge et al., 2001)

Dose (mg/kg)	*Brain Genistein Concentration, pmol/mg [µg/kg] tissue*			
	Adult		*Fetus*	
	Total	*Aglycone*	*Total*	*Aglycone*
20	0.25 **[68]**	0.22 **[59]**	0.21 ± 0.004 **[57 ± 1]**	0.19 ± 0.004 **[51 ± 1]**
34	Not reported	Not reported	Not reported	Not reported
75	Not reported	Not reported	0.23 ± 0.03 **[62 ± 8]**	0.21 ± 0.04 **[57 ± 11]**

n = 1 dam (litter) per dose group, 3–4 fetuses/litter for brain determinations. Error is SD.
From Doerge et al., 2001 *(208)*.

The authors concluded that placental transfer into fetal blood and brain probably involved the aglycone, perhaps after placental hydrolysis of conjugated forms. The higher proportion of the aglycone in the fetus was considered consistent with limited conjugation ability in the fetal rat.

Soucy *et al.*, 2006*(240)*, supported by the American Chemistry Council, evaluated genistein distribution and toxicokinetics in Crl:CD(SD) rats trated by gavage on GD 5–19 or just on GD 19 (plug=GD 0). Genistein was administered in sesame oil at 0, 4, or 40 mg/kg bw/day. Genistein, genistein glucuronide, and genistein sulfate were measured in maternal plasma, fetal plasma (pooled by litter), and placentas. Detailed results were given for the 40 mg/kg bw/day dose level (**Table 53**). Most of the genistein was present in maternal and fetal plasma as the glucuronide at both dose levels. Unconjugated genistein was the most abundant form in placenta. Genistein appeared in amniotic

fluid increasingly as the glucuronide during the 24 hours after the last dose on GD 19. Genistein was present in fetal liver largely as the glucuronide, peaking 8 hours after the last dose at about 300 µmol/kg tissue **[134 µg/kg tissue, estimated from a graph]**. Unconjugated genistein peaked in fetal brain at about 60 µmol/kg tissue **[16 µg/kg tissue, estimated from a graph]**; conjugates were present at only small amounts in brain. Genistein and its conjugates were below the limits of detection in pooled fetal reproductive organs.

Fritz *et al.*, 1998*(209)*, funded by the National Institutes of Health (NIH), treated 7-week-old female Sprague Dawley rats with dietary genistein (98.5% pure, with 1.5% methanol) at 0, 25, or 250 mg/kg diet **[ppm]**. The basal diet was AIN-76A, a phytoestrogen-free rodent feed. At 9 weeks of age, females were bred 2:1 with males that had been placed on the same diet as the females at the time of mating. Offspring were sexed at birth. Litters were standardized to 10 pups with 4–6 females. Offspring were weaned on PND 21. Genistein concentrations were determined by GC-MS in maternal serum during the lactation period (day not specified), in milk obtained by milking the dams under anesthesia, in serum from PND 7 pups (pooled by litter), in serum from PND 21 pups, and in milk from the stomach of PND 7 and 21 pups. Pup mammary tissue was also assayed for genistein on PND 7 and 21. Genistein concentrations were measured before and after incubation with β-glucuronidase/sulfatase enzymes to distinguish between free and conjugated genistein. Blood genistein levels are listed in **Table 38**, and milk and mammary gland levels are listed in **Table 54**. In serum of dams, free genistein represented 23% of genistein concentration at the low dose and 2% of the total genistein concentration at the high dose. Free genistein represented 7–33% of total genistein concentration in pup serum. **[There were no obvious patterns related to dose or age.]**

Table 54. Genistein Concentrations in Rats Fed AIN-76 Diet Supplemented with Genistein (Fritz et al., 1998)

Source	*Genistein Concentration in Feed, ppm*		
	0	*25*	*250*
Lactating Dam (PND 7 and 21)			
Milk from nipples, nM			
Total	N.D.	67 ± 10	137 ± 34
Free	N.D.	N.D.	78 ± 13
Offspring, PND 7			
Milk from stomach, nM			
Total	9 ± 2	490 ± 62	4439 ± 1109
Free	N.D.	473 ± 94	3454 ± 298
Mammary gland, nmol/kg tissue			
Total	N.D.	N.D.	440 ± 129
Free	N.D.	N.D.	318 ± 56
Offspring, PND 21			
Mammary gland, nmol/kg tissue			
Total	N.D.	0	370 ± 36
Free	N.D.	N.D.	304 ± 13

To convert nM and nmol to genistein equivalents in µg/L and µg, respectively, multiply by 0.27.
N.D.=Not determined.
From Fritz et al., 1998*(209)*.

Table 53. Toxicokinetic Data in Pregnant Rats Treated with Genistein 40 mg/kg bw/day (Soucy et al., 2006)

Source	Treatment period									
	GD 19	GD 5–19	GD 19	GD 5–19	GD 19	GD 5–19	GD 19	GD 5–19	GD 19	GD 5–19
	C_{max}, nM or ng/kg [µg/L or µg/kg]		t_{max}, hour		AUC, hour-nM or hour-ng/kg [hour-µg/L or hour-µg/kg]		Half-life, hour		Cl_{obs}, L/hour	
Maternal plasma										
Genistein	95.6 ± 2.3 **[26 ± 0.6]**	137 ± 58.8 **[37 ± 16]**	4.8 ± 5.0	3.4 ± 3.0	536 ± 112 **[145 ± 30]**	704 ± 272 **[190 ± 73]**	3.6 ± 0.9	3.9 ± 0.5	77.3 ± 46.7	60.5 ± 31.7
Glucuronide	8566 ± 1334 **[3820 ± 595]**	10438 ± 2002 **[4655 ± 893]**	1.9 ± 1.9	2.8 ± 2.2	42883 ± 34505 **[19126 ± 15389]**	65521 ± 12501* **[29222 ± 5575]**	4.3 ± 0.8	4.7 ± 1.2*	0.61 ± 0.27	0.41 ± 0.06*
Sulfate	551 ± 181 **[193 ± 63]**	557 ± 99.7 **[195 ± 35]**	1.5 ± 1.7	4.0 ± 2.3	3637 ± 2105 **[1273 ± 737]**	3557 ± 1574 **[1245 ± 551]**	4.3 ± 1.1	4.6 ± 1.3	13.2 ± 7.70	10.8 ± 3.91
Placenta										
Genistein	1088 ± 234 **[294 ± 63]**	2208 ± 282 **[596 ± 76]**	5.2 ± 2.3	6.0 ± 2.0	14040 ± 4636 **[3791 ± 1252]**	26332 ± 3952* **[7110 ± 1067]**	5.4 ± 1.2	3.0 ± 1.3	4.05 ± 1.32	2.05 ± 0.269*
Glucuronide	238 ± 104 **[106 ± 46]**	445 ± 134 **[198 ± 60]**	1.5 ± 0.7	2.6 ± 1.3	2077 ± 471 **[926 ± 210]**	4238 ± 582* **[1890 ± 260]**	5.3 ± 1.1	3.8 ± 1.0	14.8 ± 4.0	7.28 ± 0.87*
Sulfate	60.9 ± 15.7 **[21 ± 5]**	88.6 ± 14.3 **[31 ± 5]**	8.4 ± 3.6	9.6 ± 3.6	700 ± 320 **[245 ± 112]**	1211 ± 163* **[424 ± 57]**	4.8 ± 1.0	5.6 ± 2.3	64.2 ± 29.4	33.9 ± 3.80*
Fetal plasma										
Genistein	44.8 ± 5.6 **[12 ± 1.5]**	43.6 ± 8.80 **[12 ± 1.4]**	5.8 ± 4.6	4.8 ± 3.0	358 ± 120 **[97 ± 32]**	339 ± 40 **[92 ± 11]**	5.1 ± 0.9	4.2 ± 0.3	90.2 ± 1.65	64.0 ± 61.3
Glucuronide	1249 ± 247 **[557 ± 110]**	1525 ± 270 **[680 ± 120]**	7.5 ± 3.4	9.5 ± 3.0	14451 ± 1027 **[6445 ± 458]**	20346 ± 5105 **[9074 ± 2277]**	6.9 ± 1.0	7.3 ± 0.5	1.46 ± 0.06	1.76 ± 0.38
Sulfate	608 ± 254 **[213 ± 89]**	745 ± 99.4 **[261 ± 35]**	9.5 ± 3.0	9.5 ± 3.0	6940 ± 945 **[2429 ± 331**	8788 ± 3125 **[3076 ± 1094**	7.1 ± 0.7	6.9 ± 0.6	4.19 ± 0.58	5.92 ± 0.05

Data presented as mean ± SD; n not given but n = 4/data point for some figures in this study.
*Values significantly different from those obtained after single GD 19 dose.
From Soucy et al., 2006*(240)*.

The authors noted that a larger proportion of the genistein in milk from the pups' stomachs was free (78–97%) compared to milk from the dams' nipples (57%), suggesting that genistein conjugates may be hydrolyzed in the pup stomach. They also noted that the PND 21 pup data on genistein would reflect ingestion of treated maternal feed as well as transfer of genistein in milk.

Doerge *et al.*, 2000*(77)* noted that the Fritz *et al.*, 1998*(209)* study reported the proportion of total genistein in aglycone form at 72% in rat mammary gland **[82% by CERHR calculation]** (see **Table 54**). Based on this observation, Doerge *et al.*, 2006 raised the possibility of accumulation of aglycones in tissues or of hydrolysis of glycosides within tissues. **[Most likely the aglycone but not the glucuronide partitions between dam blood fat (0. 2%) and milk fat (3%) according to the lipid content of these 2 compartments, which represents a 15-fold accumulation reflected in the milk from the offspring stomachs.]** In their own study of lactational transfer of genistein, Doerge *et al.*, 2006 *(241)* placed 10 pregnant Sprague Dawley rats on a soy-free diet (5K96) until delivery, when half the dams were maintained on the basal diet and half were given feed to which genistein 500 ppm was added. Based on actual feed consumption, the genistein-treated group received a mean ± SD genistein dose of 51 ± 1.8 mg/kg bw/day. Milk was aspirated from dam nipples after oxytocin administration on PND 7 and blood was collected from dams and pups on PND 10. Conjugated and free genistein were assayed in milk and serum by LC-MS-MS. No genistein was detected in the milk of control rats. Findings in genistein-treated rats are summarized in **Table 55**. There was no correlation between PND 7 milk genistein concentration and PND 10 maternal serum genistein concentration, and there was no relationship between pup serum total or aglycone genistein concentrations and milk concentrations.

Chang *et al.*, 2000*(210)* funded by the National Center for Toxicological Research/Food and Drug Administration (NCTR/FDA), the National Institute of Environmental Health Sciences (NIEHS), and the National Toxicology Program (NTP), measured serum and tissue genistein (after enzymatic deconjugation) in Sprague Dawley rats exposed to genistein in the diet. The basal diet was an alfalfa- and soy-free diet that contained 0.54 µg/g feed **[ppm]** genistein and 0.48 µg/g **[ppm]** daidzein.

Table 55. Lactation Transfer of Genistein in Rats Given 500 ppm in Diet (Doerge et al., 2006)

Source	*Genistein Concentrations, µM [aglycone equivalent mg/L]*					
	Dam			*Pup*		
	Total	*Aglycone*	*% free*	*Total*	*Aglycone*	*% free*
Milk (PND 7)						
Mean ± SD	0.47 ± 0.21 **[0.127 ± 0.057]**	0.14 ± 0.08 **[0.038 ± 0.022]**	30			
Range	0.28–0.81 **[0.076–0.219]**	0.07–0.24 **[0.019–0.065]**	18–52			
Serum (PND 10)						
Mean ± SD	1.22 ± 1.30 **[0.329 ± 0.351]**	0.042 ± 0.037 **[0.011 ± 0.010]**	2.4[a]	0.039 ± 0.011 **[0.011 ± 0.003]**	0.001 ± 0.001 **[0.0003 ± 0.0003]**	2.6
Range	0.15–2.99 **[0.041–0.807]**	0.003–0.062 **[0.001–0.017]**	1.7–17	0.022–0.053 **[0.006–0.014]**	< LOD–0.002 **[< LOD–0.0005]**	1.2–4.6

n = 5 litters.
LOD = limit of detection.
[a] Outlier excluded by authors in calculation.
From Doerge et al., 2006*(241)*.

Treatment groups were born to female rats that (along with sires) had been exposed to genistein **[purity not specified]** at 0, 5, 100, or 500 µg/g feed (ppm) since weaning. **[Feed consumption and weight were not specified; assuming a 300 g female rat eats 30 g feed/day, additional genistein exposures would have been 0, 0.5, 10, or 50 mg/kg bw/day. A 500 g male rat eating 40 g feed/day would have been exposed to additional genistein at 0, 0.4, 8, or 40 mg/kg bw/day.]** Six litters per dose group were born to and raised by treated dams **[litter size or standardization not specified]**. Blood samples were taken from 1 pup/sex/litter at weaning on PND 21 **[plug day not specified]**, and 1 pup/sex/litter was continued on its dam's diet until PND 140. Blood samples were obtained from the tail vein 0, 4, 8, and 12 hours after removal from feed. **[It is possible that rats sampled on PND 140 were also sampled at weaning, but the methods are not clear on this issue. The method of sampling weanling rats was not indicated. On the day after tail vein sampling of PND 140 rats, these animals were killed and blood collected by cardiac puncture.]** Methanolic extracts of mammary gland, thyroid, liver, brain, and (in males) prostate and testis, or (in females) uterus and ovary were obtained from PND 140 rats and analyzed for genistein. The method of genistein analysis was LC-electrospray/MS or tandem MS.

Serum total genistein values in weanling and PND 140 rats are given in **Table 38**; values were obtained soon after removing the animals from feed, although the time of last feeding was not reported. Two-way analysis of variance (ANOVA) showed a significant effect of sex and dose on total serum genistein in PND 140 rats and an interaction of sex × dose. There was no effect of sex on serum genistein in weanling rats. The authors noted that exposure of PND 21 animals was likely through milk and through ingestion of the dams' feed ration. The authors indicate that 1–5% of genistein at both ages was unconjugated.

Genistein serum half-life and AUC for PND 140 rats are shown in **Table 56** and contrasted with the data of Coldham and Sauer *(195)*. There was a statistically significant difference between males and females for both parameters. Tissue concentrations of genistein are given in **Table 57**. There was a significant treatment effect for total genistein and genistein aglycone for all tissues. Pair-wise comparisons to controls showed elevations of total genistein in all tissues except brain in males and females fed 100 and 500 ppm genistein. Brain genistein was elevated only in the 500 ppm group. In females, ovarian, uterine, and liver total genistein concentrations were increased with 5 ppm dietary genistein compared to the control group. The authors noted that the proportion of total genistein present as the aglycone in these tissues (10–100%) was greater than the proportion in rat serum (1–5%). They also found important the differences between males and females in elimination half-life, AUC, and genistein levels in mammary gland and liver. The authors attributed the increase in

Table 56. Genistein Pharmacokinetic Parameters in PND 140 Rats Exposed to Dietary Genistein at 500 ppm (Change et al., 2000) or a Single Oral Dose of 4 mg/kg bw (Coldham and Sauer, 2000)

Sex	*Serum Half-Life, hours*		*AUC, µM-hour* [µg-hour/L][a]
	Chang et al., 2000[a]	*Coldham and Sauer, 2000*[b]	
Male	2.97 ± 0.14	12.4	22.3 ± 1.2 **[6000 ± 300]**
Female	4.26 ± 0.29	8.5	45.6 ± 3.1 **[12,000 ± 800]**

[a] Chang et al., 2000 *(210)*. Values are mean ± SEM. n = 6 or 4–6 **[both n designations appear in the paper]**.
[b] Coldham and Sauer *(195)*.

genistein in the female mammary gland to the higher lipid content in female than male mammary gland, but could not explain differences in liver genistein concentrations.

[There is an apparent contradiction between the half-life data of Chang et al., 2000*(210)* and those of Coldham and Sauer, 2000 *(195)* in Table 56; however, Coldham and Sauer used a single low dose of 4 mg/kg bw, and Chang et al., 2000 used a high daily dose rate of 50 mg/kg bw. Another difference is that the rats in Change et al., 2000 were dosed continuously with genistein in the diet and sequential plasma samples obtained after withdrawal from food, i.e., no absorption or distribution phases complicated the analysis. Greatly decreased half-life at high dose rates is probably due at least in part to saturation of glucuronidation and, hence, reduced enterohepatic circulation. At high genistein dose rates, 17β-estradiol cannot displace genistein from plasma protein binding anymore. It can be expected that a much smaller portion of the higher dose would be bound to plasma proteins, contributing to the lower half-life. The reversal of male:female half-life ratios at high daily dose rates is probably due to differential maximum velocity (V_{max}) of various intestinal and possible hepatic UDPGTs.]

In a thyroid toxicity study was conducted with similarly treated rats, Chang *et al.*, 2000*(242)* noted that higher levels of aglycone in thyroid suggested that non-polar aglycones preferentially partition into lipophilic tissues.

Lewis *et al.*, 2003*(243)*, funded by UK Foods Standards Agency, evaluated milk and serum concentrations of genistein in rats **[strain not specified]** as part of a study on developmental effects of lactation period exposure (reviewed in Section 3). Genistein (98.3% purity) was given to 4 lactating rats at a single oral dose of 16 mg/kg bw. Litter size was reduced to 6 after spontaneous delivery. Milk and plasma samples were taken every 24 hours for 5 days **[method of collection not specified]**. One pup/litter/day was killed and blood obtained for analysis. The experiment was repeated using ^{14}C-genistein at 50 mg/kg bw. Genistein was quantified by LC with an ultraviolet detection system. Genistein metabolites were characterized by LC-MS following enzymatic digestion with β-glucuronidase/sulfatase. In an additional study, rat pups were dosed directly with either sc or oral genistein (either unlabelled or ^{14}C-labeled) on PND 7. Doses were 0, 0.4, 4, or 40 mg/kg, given once, with cohorts of animals killed and blood collected at 0.5, 1, 2, 4, 6, 8, 12, and 24 hours after dosing. Quantification was by LC with ultraviolet detection.

The maximum concentration of genistein in maternal plasma was 180 µg/L **[665 nM]** without β-glucuronidase/sulfatase pretreatment and 1800 µg/L **[6651 nM]** after enzyme pretreatment. Time to peak plasma genistein in maternal plasma was 8 hours without enzyme pretreatment and 2 hours with enzyme pretreatment. Milk genistein peaked 1–3 hours after dosing at 40 µg/L **[148 nM]** for untreated milk and at 170 µg/L **[628 nM]** for enzyme-pretreated milk. After administration of 50 mg/kg bw radiolabeled genistein, peak plasma, erythrocyte, and milk genistein levels obtained in dams at 8 hours were 7100 µg equivalents **[26,235 nmol]**/kg in plasma, 800 µg equivalents **[2956 nmol]**/kg in erythrocytes, and 3700 µg equivalents **[13,672 nmol]**/kg in milk. Pup genistein peaked 24 hours after maternal dosing at 100 µg equivalent **[370 nmol]**/kg for both plasma and erythrocytes. The authors interpreted the results as showing that secretion of genistein into milk is approximately 0.04% of the maternal dose at 8 hours. Plasma concentrations after direct administration of genistein to PND 7 pups are shown in Table 58.

[There is an apparent contradiction between the report of Fritz et al., 1998*(209)* and the data provided by Lewis at al.*(243)* and Doerge et al., 2006*(241)* regarding milk content of genistein and/or derivatives.

Table 57. Tissue Genistein Levels in PND 140 Rats Exposed to Dietary Genistein (Chang et al., 2000)

Tissue		Dietary genistein, ppm							
		0		5		100		500	
		Genistein Level, pmol/mg tissue [µg/kg tissue genistein equivalent]							
		Male	Female	Male	Female	Male	Female	Male	Female
Mammary	Aglycone	N.D.	N.D.	N.D.	N.D.	0.16 ± 0.04 **[44 ± 11]**	0.12 ± 0.02 **[32 ± 5]**	0.20 ± 0.04 **[54 ± 11]**	1.18 ± 0.22 **[319 ± 59]**
	Total	0.020 ± 0.004 **[5 ± 1]**	0.02 ± 0.04 **[5 ± 11]**	0.020 ± 0.002 **[5 ± 0.5]**	0.030 ± 0.004 **[8 ± 1]**	0.33 ± 0.05 **[89 ± 14]**	0.29 ± 0.06 **[78 ± 16]**	0.83 ± 0.16 **[224 ± 43]**	2.39 ± 0.34 **[646 ± 92]**
Thyroid	Aglycone	0.040 ± 0.01 **[11 ± 3]**	0.04 ± 0.014 **[11 ± 4]**	0.060 ± 0.07 **[16 ± 19]**	0.043 ± 0.020 **[12 ± 5]**	0.060 ± 0.01 **[16 ± 3]**	0.076 ± 0.008 **[21 ± 2]**	0.11 ± 0.03 **[30 ± 8]**	0.212 ± 0.04 **[57 ± 11]**
	Total	0.090 ± 0.01 **[24 ± 3]**	0.047 ± 0.009 **[13 ± 2]**	0.10 ± 0.11 **[27 ± 30]**	0.061 ± 0.012 **[16 ± 3]**	0.22 ± 0.03 **[59 ± 8]**	0.277 ± 0.052 **[75 ± 14]**	0.41 ± 0.08 **[111 ± 22]**	1.15 ± 0.23 **[310 ± 62]**
Liver	Aglycone	< 0.02 **[< 5]**	0.01 **[3]**	< 0.02 **[< 5]**	0.06 ± 0.01 **[16 ± 3]**	0.02 **[5]**	1.07 ± 0.21 **[289 ± 57]**	0.23 ± 0.08 **[62 ± 22]**	5.66 ± 1.31 **[1528 ± 354]**
	Total	< 0.02 **[< 5]**	0.02 **[5]**	< 0.02 **[< 5]**	0.12 ± 0.01 **[32 ± 3]**	0.32 ± 0.10 **[86 ± 27]**	1.68 ± 0.39 **[454 ± 105]**	0.67 ± 0.14 **[181 ± 38]**	7.33 ± 1.62 **[1979 ± 437]**
Brain	Aglycone	< 0.02 **[< 5]**	< 0.02 **[< 5]**	< 0.02 **[< 5]**	N.D.	< 0.02 **[< 5]**	N.D.	0.04 **[11]**	0.03 **[8]**
	Total	< 0.02 **[< 5]**	< 0.02 **[< 5]**	< 0.02 **[< 5]**	N.D.	< 0.02 **[< 5]**	N.D.	0.04 **[11]**	0.06 **[16]**
Prostate	Aglycone	0.020 ± 0.006 **[5 ± 2]**	N/A	N.D.	N/A	0.40 ± 0.13 **[108 ± 35]**	N/A	0.49 ± 0.18 **[132 ± 49]**	N/A
	Total	0.020 ± 0.003 **[5 ± 0.8]**		0.030 ± 0.003 **[8 ± 0.8]**		0.80 ± 0.23 **[216 ± 62]**		1.09 ± 0.23 **[295 ± 62]**	
Testis	Aglycone	0.020 ± 0.003 **[5 ± 0.8]**	N/A	N.D.	N/A	0.40 ± 0.006 **[108 ± 2]**	N/A	0.07 ± 0.01 **[19 ± 3]**	N/A
	Total	0.030 ± 0.01 **[8 ± 3]**		0.040 ± 0.004 **[11 ± 1]**		0.42 ± 0.08 **[114 ± 22]**		0.63 ± 0.12 **[170 ± 32]**	
Ovary	Aglycone	N/A	N.D.	N/A	N.D.	N/A	0.40 ± 0.04 **[108 ± 11]**	N/A	0.85 ± 0.09 **[230 ± 24]**
	Total		0.010 ± 0.002 **[3 ± 0.5]**		0.059 ± 0.026 **[16 ± 7]**		0.42 ± 0.05 **[114 ± 14]**		1.07 ± 0.11 **[289 ± 30]**
Uterus	Aglycone	N/A	N.D.	N/A	N.D.	N/A	0.64 ± 0.07 **[173 ± 19]**	N/A	1.43 ± 0.33 **[386 ± 89]**
	Total		0.010 ± 0.001 **[3 ± 0.3]**		0.037 ± 0.006 **[10 ± 2]**		0.78 ± 0.11 **[211 ± 30]**		1.42 ± 0.27 **[384 ± 73]**

Values are mean ± SEM, n = 6/sex/dose group. The number of significant figures is as in the original.
The conversion to pg/mg tissue was rounded to the nearest integer if higher than 1.
N/A = Not applicable; N.D. = Not done.
From: Chang et al., 2000 *(210)*.

Table 58. Plasma Concentration of Total Radioactivity after Administration of Single Doses of [^{14}C] Genistein to Rat Pups on PND 7 (Lewis et al., 2003)

Hours After Dose	Dose, mg/kg bw					
	0.4	4[a]	40	0.4	4[a]	40
	Male			Female		
Oral Administration						
2	82 ± 21 **[303 ± 78]**	910 **[3367]**	19,400 ± 2080 **[71,788 ± 7697]**	89 ± 22 **[329 ± 81]**	761 **[2816]**	9360 ± 2190 **[34,636 ± 8104]**
4	20 ± 5 **[74 ± 19]**	230 **[851]**	2550 ± 659 **[9436 ± 2439]**	24 ± 8 **[89 ± 30]**	189 **[699]**	5040 ± 1680 **[18,650 ± 6217]**
8	26 ± 1 **[96 ± 3.7]**	**251** **[929]**	1790 ± 102 **[6623 ± 377]**	42 ± 21 **[155 ± 78]**	272 **[1007]**	1650 ± 223 **[6106 ± 825]**
AUC[b]	460 **[1702]**	4580 **[16,948]**	56,800 **[210,184]**	790 **[2923]**	4760 **[17,614]**	46,300 **[171,329]**
SC Administration						
2	177 ± 24 **[655 ± 89]**	834 **[3086]**	7630 ± 1580 **[28,234 ± 5847]**	163 ± 26 **[603 ± 96]**	1140 **[4218]**	9070 ± 1130 **[33,563 ± 4181]**
4	86 ± 12 **[318 ± 44]**	634 **[2346]**	5130 ± 388 **[18,983 ± 1436]**	132 ± 9 **[488 ± 33]**	1160 **[4292]**	7120 ± 696 **[26,347 ± 2575]**
8	63 ± 12 **[233 ± 44]**	171 **[633]**	2550 ± 1430 **[9436 ± 5292]**	90 ± 3 **[333 ± 11]**	588 **[2176]**	2540 ± 307 **[9399 ± 1136]**
AUC[b]	780 **[2886]**	5320 **[19,686]**	38,300 **[141,726]**	970 **[3589]**	7520 **[27,827]**	48,100 **[177,990]**

Data expressed as µg genistein equivalents/L **[nM].**
Mean ± SD, n = 4.
[a] SD not given for 4 mg/kg dose.
[b] AUC expressed in µg equivalents-hour/L **[nM equivalents-hour]**.
From Lewis et al., 2003*(243)*.

Whereas Lewis et al., 2003 reported finding metabolites of genistein only in milk of dams given a single dose of ^{14}C-genistein, Fritz et al., 1998 recovered mainly the parent compound from the stomach milk of pups. Fritz et al., 1998 administered genistein in the diet (500 ppm ≈ 50 mg/kg bw/day) and, therefore, genistein was at steady state, whereas a single genistein dose of 50 mg/kg bw given by Lewis et al., 2003 resulted in undetectable plasma levels after 24 hours. As discussed above, daily dosing with high dose rates of genistein over prolonged periods of time reduced the half-life of genistein dramatically, probably as a result of increased free fraction of the parent compound over the glucuronide. At steady state, equilibration between plasma and milk does occur, but not after a single dose, which is the most likely explanation for the observed discrepancy.] According to data available in abstract form, administration of 40 mg/kg bw genistein on GD 19 to pregnant rats resulted in fetal:maternal plasma ratios of 0.25 for genistein, 0.04 for genistein-7-O-glucuronide, 0.05 for genistein-4-O-glucuronide, and 0.55 for sulfate conjugates at 1 hour following dosing *(244)*.

2.1.2.3 Metabolism

As in humans, most genistein in rats is conjugated with glucuronic acid by UDPGT prior to entering the systemic circulation. A study examining the ontogeny of UDPGT in rats *(245)* is presented in **Section 2.5**.

Sfakianos *et al.*, 1997 *(246)* conducted a series of studies in female Sprague Dawley rats to determine intestinal uptake and biliary excretion of genistein. The rats were fed soy- and isoflavone-free diets prior to the studies. During the studies, rats were anesthetized and ^{14}C-labeled genistein was infused into the intestine or portal vein. Bile, sera, and serosal fluids were collected over periods of up to 4 hours following infusion. One to 3 rats were used in analyses to measure genistein and metabolite levels in body fluids or perfusates. When ^{14}C-genistein was infused into isolated duodenum, radioactivity appeared in bile within 20 minutes and reached equilibrium within 1 hour; biliary output of genistein metabolites decreased from 9.2 to 7.7 to 6.7% when the infusion rate was increased from 62 to 124 to 247 nmol/hour **[17 to 34 to 67 µg/L/hour]**. When genistein was infused into the duodenum and allowed to proceed down the intestinal tract, radioactivity peaked in bile within 80 minutes, thus demonstrating efficient intestinal uptake and biliary excretion; a total of 70–75% of the dose was recovered in bile within 4 hours. Analyses using HPLC-MS or HPLC following β-glucuronidase treatment confirmed that the primary metabolite in bile was genistein glucuronide. When collected bile was pooled, diluted, and reinfused into the duodenum or ileum, radioactivity was immediately detected in bile and continued to increase during the remaining 4-hour period (data not shown by study authors). In studies in which ^{14}C-genistein was infused into the portal vein, efficient glucuronidation by liver and biliary excretion was demonstrated. Only genistein glucuronide was detected in peripheral blood when the infusion rate into portal vein was 0.77 nmol/minute **[0.21 µg/minute]**, while both genistein glucuronide and genistein were detected in peripheral blood at an infusion rate of 8.82 nmol/minute **[2.4 µg/minute]**. Although glucuronidation by liver was demonstrated, collection of blood from the portal vein of a rat following a 1-hour duodenal infusion with ^{14}C-genistein revealed that most of the radioactivity was represented by genistein glucuronide, thus indicating that glucuronidation occurs within the intestinal wall. To verify glucuronidation by the intestinal wall, everted intestinal sac preparations were filled with a solution containing 27 µM **[7297 µg/L]** genistein and incubated for 3 hours; both genistein and genistein glucuronide were detected inside the intestinal sac preparations. Based on the findings of this study, the study authors concluded that genistein undergoes efficient enterohepatic circulation. Glucuronidation within the intestinal wall was also demonstrated.

The study by Coldham and Sauer, 2000 *(195)* reported that in adult rats gavaged with 4 mg/kg bw ^{14}C-genistein, the major metabolites in plasma were genistein glucuronide and 4-hydroxyyphenyl-2-propionic acid, while parent compound was present at trace levels. Major urinary metabolites identified in this and previous studies in rats included genistein glucuronide, dihydrogenistein glucuronide, genistein sulfate, dihydrogenistein, 6′-hydroxy-*O*-desmethylangolensin, and 4-hydroxyphenyl-2-propionic acid. All metabolites except 4-hydroxyphenyl-2-propionic acid have also been identified in humans, suggesting common pathways in rats and humans. As in humans, genistein glucuronide was the most abundant plasma metabolite in rats. Parent compound was the predominant form of genistein in uterus, while in prostate the most abundant form was the metabolite 4-hydroxyphenyl-2-propionic acid.

Blood profiles of genistein in Sprague Dawley rats dosed with genistein in diet as part of a dose range-finding study for a two-generation study are summarized in **Table 38** *(213)*. Most of the genistein in adult rats was present as glucuronide conjugates. A small percentage of total genistein was represented by aglycone **[1.4–2.9%]** and sulfate conjugates **[<1.0–7.3%]**. **[Glucuronide levels exceeded total genistein levels.]** Two different glucuronide conjugate isomers were identified: 4′-glucuronide and 7′-glucuronide.

Lund *et al.*, 2001 *(247)* also reported significant production of equol in rats fed a soy-based diet. Serum total equol levels **[~3000–4000 ng/mL; 12,389–16,519 nM]** that were about an order of magnitude

higher than levels of total genistein **[~300–500 ng/mL; 1110–1850 nM]** or daidzein **[~300–400 ng/mL; ~1180–1573 nM]** in adult rats fed soy-based diets containing phytoestrogens at 600 µg/g diet. In a review, Setchell *(64)* reported that high levels of equol equivalents have been detected in portal venous blood and bile of rats, thus indicating that isoflavones undergo enterohepatic circulation. Because equol was primarily found as a conjugate of glucuronic acid in venous portal blood, it was suggested that conjugation within the intestinal wall may occur during absorption.

A k_m value of 7.7 µM **[2081 µg/L]** and V_{max} value of 1.6 µmol **[432 µg]**/mg protein-minute were reported for formation of genistein glucuronide following *in vitro* incubation of genistein with rat liver microsomes *(248)*.

Species differences in metabolic profiles

Gu *et al.*, 2006 *(176)* compared the isoflavone profiles for genistein, daidzein, equol, and glycitein and other isoflavone metabolites in women, female Sprague Dawley rats, Hamphire/Duroc Cross pigs, and cynomolgus monkeys following ingestion of soy protein isolate (results are summarized in **Table 59**). In addition to equol, other metabolites measured were *O*-DMA, dihydrogenistein, and dihydrodaidzein. The dietary intake of soy protein (and isoflavones), the duration of soy intake, and age differed among experiments which precluded direct comparison of serum or urinary isoflavone concentrations. For genistein, administered doses ranged from 1 to 13 mg/kg, and for daidzein the range was 0.6 to 9.9 mg/kg. The authors conducted a second rat experiment where dose, duration, and postprandial time course were matched in young adult female rats (n=4) to those of the female human subjects. In that study, rats were administered a bolus of soy protein isolate through an intragastric cannula that provided 1.0 mg/kg genistein, 0.6 mg/kg daidzein, and 0.1 mg glycitein. Blood samples were collected through a femoral cannula at 4 hours post-dose. Total genistein, daidzein, and glycitein and metabolites were also measured in 3 breastfed infant rhesus monkeys as 2, 4, and 5 months of age to assess the onset of ability to produce equol. Urinary and serum isoflavone aglycones were measured by LC-MS before and after enzymatic deconjugation with sulfatase and glucuronidase to obtain unconjugated and total isoflavone concentrations. Samples were also incubated with β-glucuronidase to obtain the sum of glucuronides and aglycones. Isoflavone sulfates were calculated by subtracting the glucuronides and aglycones from the total concentration. Equol was measured by electrochemical detection.

The authors reported significant interspecies differences in metabolism with pigs having an overall metabolic profile that was the most similar to women. This conclusion was based primarily on species differences in equol production. Equol was detected in considerably higher serum and urine concentrations in rats and monkeys compared to women and pigs. For example, serum equol measured in rats and monkeys represented 77 and 52% of the summed serum isoflavones but were undetectable in pigs and women. Genistein and daidzein were the major contributors to summed serum isoflavones in pigs (88%) and women (91%). Similar patterns were observed for urine. The equol contribution to the summed isoflavones in pigs, monkeys, and rats was 2%, 51%, and 69%. Equol was not detected in the urine of women**.** Genistein and daidzein contributed 86%, 38%, 28%, and 81% to the summed isoflavones in pigs, monkeys, rats, and women. The same pattern was observed when soy protein dose was matched in rats to the dose administered to women (**Table 60**). Plasma concentrations of genistein and daidzein were 11-fold and 4-fold higher in women compared to rats and equol was not detected in the plasma from urine. The other most apparent difference across species was the relative abundance of aglycone in urine (**Table 59**). In pigs and women, < 10% of genistein and daidzein detected in urine were aglycones.

Table 59. Proportion of Isoflavone Conjugates in Animal Serum, Human Plasma, and Urine after Consumption of Diets Containing Soy Protein Isolate (Gu et al., 2006)

Isoflavone and Species[a]	Intake, mg/kg	Duration	Animal Serum or Human Plasma, %			Urine, %		
			Aglycone	Glucuronide	Sulfate	Aglycone	Glucuronide	Sulfate
Genistein								
Rats	13	3–4 days	3.6	50.4	45.9	46.9	41.6	11.5
Monkeys	4.8	1 day	3.5	23.8	72.8	89.2	5.6	5.2
Pigs	10.9	1 day	2.2	48.7	49.0	6.0	81.6	12.4
Women	1	1 day	1.2	78.4	20.4	0.1	86.7	13.3
Daidzein								
Rats	9.9	3–4 days	7.3	68.2	24.5	40.8	47.3	11.9
Monkeys	3.7	1 day	0.6	34.5	64.9	90.9	6.4	2.7
Pigs	8.6	1 day	5.0	73.3	21.6	4.3	85.6	10.0
Women	0.6	1 day	1.4	75.1	23.5	0.3	86.1	13.6
Equol								
Rats	N/A		0.7	92.6	6.7	32.8	65.5	1.8
Monkeys			6.1	29.6	64.2	96.3	3.7	0.0
Pigs								
Women								

[a] Sample sizes were: rats (n=7–9), monkeys (n=6–8), pigs (n=5), and women (n=6).
N/A=Not applicable.
From Gu et al., 2006 *(176)*.

Table 60. Isoflavones and Metabolites in the Plasma of Women and Rat Serum after a Matched Single Dose of Soy Protein Isolate (Gu et al., 2006)

Isoflavone and Species[a]	*Intake, mg/kg*	*Concentration, µmol/L*
Genistein		
Rats	1.0	~0.15
Women	1.0	~1.15
Daidzein		
Rats	0.6	~0.20
Women	0.6	~0.70
Glycitein		
Rats	0.1	–
Women	0.1	~0.08
Equol		
Rats	N/A	~0.5
Women		–
DMA		
Rats	N/A	~0.13
Women		~0.08
Dihydrogenistein		
Rats	N/A	< ~0.05
Women		< ~0.05
Dihydrodaidzein		
Rats	N/A	~0.10
Women		~0.08

[a]Sample sizes were n=4 rats and n=10 women.
– = not detectable; N/A=Not applicable.
From Gu et al., 2006 *(176)*.

In contrast, much higher percentages of genistein and daidzein present as aglycones were detected in rat urine (41 to 47%) and monkey urine (89 to 91%). Much smaller differences were observed for the percent as aglycone in animal serum or human plasma. For genistein, 3.6%, 3.5%, 2.2%, and 1.2% was present as aglycone in rats, monkeys, pigs, and women. For daidzein, the percent present as aglycone in rats, monkeys, pigs, and women were 7.3%, 0.6%, 5.0%, and 1.4%, respectively.

Consistent with other studies, a relatively small percentage of daily isoflavone dose was excreted in 24-hour urine samples. Based on urinary concentrations of total genistein and dihyrogenistein, the percent of genistein dose excreted was 2.6%, 44.9%, and 11.8% in rats, pigs, and women. Daidzien excretion was based on urinary concentrations of total daidzein, equol, *O*-DMA, and dyhydrodaidzein. The percent of daidzein dose excreted was 21.2%, 46%, and 55% in rats, pigs, and women. The percent of glycitein dose excreted in rats, pigs, and women were 4.7%, 17.0%, and 18.1%, respectively.

By six months of age, serum equol accounted for 80% of summed isoflavones in breastfed infant rhesus monkeys, leading the authors to conclude that infant monkeys are ingesting feed consumed by the adults in the colony by this age and that the onset of equol production occurs early in monkeys.

2.1.2.4 Elimination

Coldham and Sauer, 2000 *(195)* conducted a mass-balance study where rats (n=5/sex) were housed in metabolism cages and urine and feces collected at 4, 8, 12, 24, 48, 72, 96, 120, 144, and 168 hours after being gavaged with 4 mg/kg bw ^{14}C-genistein (**Table 61**). About 90% of the dose was recovered within 48 hours following dosing. Elimination half-life was 12.4 hours in males and 8.5 hours in females. Total clearance was 1.18 mL/minute in males and 2.0 mL/minute in females. In pregnant rats treated by gavage with genistein 40 mg/kg bw/day on GD 5–19, mean ± SD plasma clearance of unconjugated genistein was 64.0 ± 61.3 L/hour *(240)*

Table 61. Average Mass Balance in Male and Female Rats Following Oral Dosing with 4 mg/kg [^{14}C] Genistein (Coldham and Sauer, 2000)

Sex	Mass Balance, % dose				
	Urine	Feces	Carcass	Cage Washings	Recovery
Male	67.3	30.6	0.2	3.7	101.8
Female	66.4	36.0	0.6	1.1	104.1

n=5/sex.
From Coldham and Sauer, 2000 *(195)*.

Goelzer *et al.*, 2001 *(230)* characterized the mass balance of 3-(^{14}C)-genistein administered as a single oral gavage dose of 5 or 50 mg/kg to male and female rats. To determine mass-balance, urine and feces were collected for 120 hours (n=5/sex/dose level); radioactivity was determined in the excreta and carcasses. The elimination of radioactivity was rapid with >78% of the administered dose being recovered in urine and feces within 24 h of dose administration (see **Table 62**). The major route of elimination of radioactivity was renal excretion, indicating that absorption of (^{14}C)-genistein was extensive. In biliary cannulated animals, 30 to 50 % of the dose was excreted in the bile. Presumably

Table 62. Excretion of Radioactivity Following Oral Administration of [^{14}C] Genistein to Rats at Dose Levels of 5 and 50 mg/kg (Goelzer et al., 2001)

Collection Time (h)	Urine, % administered dose				Feces, % administered dose			
	5 mg/kg		50 mg/kg		5 mg/kg		50 mg/kg	
	Male	Female	Male	Female	Male	Female	Male	Female
6	13.16	13.84	4.48	5.32	–	–	–	–
12	35.01	33.83	33.57	21.76	–	–	–	–
24	30.43	20.08	26.34	24.10	20.59	17.72	21.97	27.36
24 hour total	***78.60***	***67.75***	***64.39***	***51.18***	***20.59***	***17.72***	***21.97***	***27.36***
48	1.98	1.86	1.16	1.36	3.60	2.93	3.12	2.00
72	0.24	0.24	0.15	0.19	0.25	0.37	0.19	0.23
96	0.12	0.10	0.15	0.11	0.06	0.05	0.06	0.04
120	0.06	0.05	0.03	0.04	0.03	0.04	0.02	0.02
Total	***81.00***	***70.00***	***65.89***	***52.90***	***24.53***	***21.11***	***24.34***	***29.65***

From Goelzer et al., 2001 *(230)*.

the radioactivity represents genistein conjugates as the main portion was recovered within 12 h after dosing. Renal excretion fell by 30 to 50% suggesting significant entero-hepatic circulation, which is supported by the pharmacokinetic data. Fecal elimination was also reduced considerably indicating that biliary excretion was also an important route of elimination. Absorption of radioactivity, estimated from the extent of urinary and biliary excretion, including radioactivity detected in the cage washes, was >87% of the administered dose emphasizing that absorption of (14C)-genistein was significant after oral administration. There was no noticeable sex difference in the excretion of radioactivity and a ten-fold increase in dose administered had no noticeable effect on the rate or routes of excretion.

2.2 General Toxicology and Biological Effects

2.2.1 General Toxicity Studies

McClain *et al.*, 2006 *(229)* conducted a series of studies to examine toxicity of genistein in rats **[Funding support not indicated. Authors are affiliated with McClain Associates, DSM Nutritional Products Ltd., or Hoffman-La Roche Ltd]**. Two acute studies were conducted in male and female 7-week-old Hanlbm Wistar rats and 8-week-old outbred Wistar Crl:(WI)BR rats. The Hanlbm Wistar rats were fed a genistein-free diet and the Wistar Crl:(WI)BR rats were fed standard animal diet. The rats were administered genistein (99.5–99.6% purity) in a single gavage dose of 2000 mg/kg bw and observed for 2 weeks. The rats were then killed and necropsied. Liver and kidney weights were measured in the Hanlmb rats. **[The number of rats treated and observed was not stated.]** All rats survived, and there were no gross effects at necropsy or changes in organ or body weights. In the Wistar Crl:(WI)BR rats, lethargy was noted in all males and 1 female on "day 1" and alopecia was observed on "days 14 and 15." The study authors concluded that genistein has low acute toxicity.

In subchronic and chronic studies conducted by McClain *et al.*, 2006 *(229)* outbred Wistar rats were fed diets containing genistein for 4, 13, or 52 weeks. Assuming exposures started immediately following a 1-week acclimation period, rats were 7 weeks old in the 4- and 13-week studies and 5 weeks old in the 52-week study at the start of dosing. Purity of genistein was reported at 99% for the 4-week study and ≥99.4–99.8% for the 13-and 52-week studies. Dietary genistein concentrations were adjusted weekly to obtain target dose. Diets were assessed for homogeneity and stability of genistein. The 13- and 52-week studies were conducted according to Good Laboratory Practice (GLP). Body weight and feed intake were measured. Ophthalmology, clinical chemistry, hematology, and urinalyses parameters were examined near the end of the exposure period in the 4- and 13-week studies, every 13 weeks in the 52-week study, and following recovery periods. Rats were killed and necropsied following treatment or recovery periods. Organ weights were recorded and histopathological analyses were conducted at the end of treatment periods and following recovery periods. Levels of free and total genistein were measured in plasma, kidney, and liver in the 4- and 13-week studies and in plasma at 26 and 52 weeks of exposure. According to the study authors, blood levels of total genistein at 5, 50, and 500 mg/kg/day at 52 weeks were equivalent to ~4, 22, and 143 times human exposure levels. Percentages of free and total genistein in blood and tissues are reported in **Secion 2.2.1**. Statistical analyses included Dunnett test, Steel test, and Fisher exact test.

In the 4-week dose range-finding study, 6 rats/sex/group were fed diets providing genistein doses of 0, 0.5, 5, 50, or 500 mg/kg/day genistein. No data were presented by study authors for the 4-week study, and thus there is insufficient information for Expert Panel review. Briefly, the study did not

detect treatment-related effects on mortality, clinical signs, or ophthalmological parameters. Body weight gain was reduced in males and females of the 500 mg/kg/day group.

Non-dose related decreases in red blood cell counts, slightly decreased hemoglobin and hematocrit values, and slightly increased reticulocyte counts in high-dose females were the only hematological effects reported. Clinical chemistry findings included increased triglycerides, phospholipids, calcium, phosphorus, and chloride in males and decreased uric acid and increased total protein in females. **[Doses at which effects occurred were not stated.]** Increases in adrenal weight of males and relative liver, kidney, spleen, ovary, and uterus weights of females in the 500 mg/kg/day group were the only organ weight effects that authors considered treatment related. Reduced seminal vesicle size was observed at necropsy in 3 of 6 males from the 500 mg/kg/day group. No treatment-related organ lesions were reported.

In the 13-week study that was conducted according to GLP, 15 rats/sex/group were fed diets containing genistein doses of 0, 5, 50, or 500 mg/kg/day. Following treatment, 10 rats/sex/group were killed and 5 rats/sex/group were allowed to recover for 4 weeks to determine reversibility of treatment-related effects. No treatment-related deaths were observed. Body weights were lower in the 500 mg/kg/day group compared to the control group **[18% lower for males and 10% lower for females]**. Body weights of males increased during the recovery period but were still lower compared to controls at the end of the study. During the first month of treatment, feed intake was reduced in male rats of the 500 mg/kg/day group. Hematology, clinical chemistry, and urinalysis parameters were monitored following 11 weeks of treatment **[data were not shown]**. Red blood cell parameters were reportedly decreased and reticulocyte levels were increased in males and females of the 500 mg/kg/day group. Slight changes in clinical chemistry parameters included decreased glucose and increased uric acid, sodium, and chloride in high-dose males and decreased uric acid and increased calcium, total protein, and phospholipid in high-dose females. Uric acid crystals were increased in females of the 500 mg/kg/day group. Non-reproductive organ weight changes in high-dose males included slight increases in relative (to body weight) heart, thyroid, kidney, and adrenal weights. Relative to body weight, testis weights was increased **[by 19%]** in high-dose males **[possibly due to decreased body weight]**. Relative liver and kidney weights were increased in females of the 500 mg/kg/day group. Relative uterine weight of high-dose females was increased **[by 41%]**. **[The study authors did not present data for non-reproductive organ weights.]** All animals were necropsied, and histopathological evaluations were conducted in tissues from control and high-dose animals. There were no treatment-related gross or histopathological alterations. Ophthalmologic parameters were also unaffected. With the exception of body weight effects in males, none of the treatment-related effects were observed following the 4-week recovery period. **[No recovery data were reported by study authors.]**

In the 52-week study that was conducted according to GLP, 30 rats/sex/group were fed diets providing genistein doses of 0, 5, 50, or 500 mg/kg/day. Five rats/sex/group were killed following 26 weeks of treatment and 20 rats/sex/group were killed following 52 weeks of treatment. Five rats/sex/group were allowed to recover for 8 weeks during which time they received no treatment. There were no treatment-related deaths during the study. A higher rate of alopecia in male and female rats of the high-dose group was the only clinical sign of toxicity reported. No effects were noted for ophthalmologic parameters. Body weight gain was reduced in high-dose male and female rats from the week 26 of treatment through the week 1 of recovery. During that time period body weights of high-dose animals compared to control animals were ~30–35% lower for males and ~30% lower for females; $P<0.01$. Feed intake was reduced by 22% in males and females of the high-dose group but was not statistically different when analyzed on a

weekly basis. A number of statistically significant effects on hematology and clinical chemistry parameters were observed. The effects that the authors considered treatment-related in high-dose animals are listed in Table 63 along with magnitudes of change observed and the weeks for which the effects were observed. Other statistically significant effects on hematology and clinical chemistry were observed, but the authors considered the effects to be incidental because there were either no dose-response relationships or values were within normal ranges. Some of the hematological effects persisted through the recovery period, but all clinical chemistry effects were resolved during recovery. Organ weights were measured at weeks 26 and 52. The only significant organ weight effects that the authors considered to be treatment-related at 52 weeks were increased relative weights of adrenal and spleen (males and females), prostate [47%], testis [52%], ovary [394%], and uterus [275%] in the 500 mg/kg/day group. Increases in adrenal, spleen, and uterus weights were also observed following 26 weeks of treatment. Increased ovary weight was the only organ weight effect that persisted through the recovery period. Other significant organ weight effects occurred, but the study authors concluded that those effects resulted from reduced body weight gain.

Table 63. Hematological and Clinical Chemistry Effects Observed in Rats Treated with Genistein 500 mg/kg/day (McClain et al., 2006)

Parameter	Males		Females	
	Effect	Weeks Effect Observed	Effect	Weeks Effect Observed
Hematology				
Red blood cell count	↓4–6%	13, 26, recovery	↓4–5%	13, 26
Mean corpuscular volume	↑4–10%	13, 26, 52, recovery	↑2%	13
Mean corpuscular hemoglobin	↑3–11%	13, 26, 52, recovery	↔	
Reticulocyte count	↑18%	13	↑16–36%	13, 26, recovery
White blood cell count	↓14%	13	↔	
Hemoglobin	↔		↓4%	13, 26
Mean corpuscular hemoglobin concentration	↔		↓1–3%	13, 52
Clinical chemistry				
Bilirubin	↓22–23%	13, 26	↓18–20%	13, 26, 52
Creatinine	↓6%	13, 26, 52	↔	
Cholesterol	↓3–50%	13, 26, 52	↔	
Glucose	↓12–29%	26, 52	↔	
Protein	↓4–5%	13, 26, 52	↔	
γ-Glutamyl transferase	↑50–53%	13, 26	↑46–61%	13, 26
Uric acid	↓58%	13	↑45–55%	13, 26
Lactate dehydrogenase	↔		↑22–67%	26, 52
Alkaline phosphatase	↔		↑19–27%	13, 26, 52

↓,↑,↔ Statistically significant decrease, increase or no statistically significant or treatment-related effect.
From McClain et al., 2006 *(229)*.

At the 52-week necropsy, uterine horn dilation was observed in 7 females of the 500 mg/kg/day group and watery cysts in ovaries were noted in 4, 3, and 12 females of the low-, mid-, and high-dose group. **[It is assumed that ~20 females/dose group were examined.]** Genistein-related histopathology was observed at 26 and 52 weeks, and the effects and incidences at 52 weeks are summarized in Table 64 for males and Table 65 for females. In male rats, epididymal vacuolation was observed at 500 mg/kg/day and prostate

Table 64. Treatment-Related Histopathological Effects in Male Rats Given Genistein in Diet for 52 Weeks (McClain et al., 2006)

Effect	*Animals Affected/Animals Examined at each Genistein Dose, mg/kg/day*				*Benchmark dose, mg/kg/day*[a]	
	0	*5*	*50*	*500*	BMD_{10}	$BMDL_{10}$
Epididymal vacuolation	6/19	8/20	9/20	11/20		
Prostate inflammation	6/20	2/20	14/20[b]	18/20[b]	48	34
Fatty change in liver	16/20	15/20	17/20	8/20[c]	120	84
Bile duct proliferation in liver	3/20	3/20	2/20	6/20		
Osteopetrosis	0/20	0/20	0/20	17/20[b]	346	145

[a] The BMD10 is the benchmark dose associated with a 10% effect, estimated from a curve fit to the experimental data. The BMDL10 represents the dose associated with the lower 95% confidence interval around this estimate. Benchmark doses are used commonly in a regulatory setting; however, they are used in this report when the underlying data permit their calculation, and are only supplied to provide 1 kind of description of the dose-response relationshIP in the underlying study. Calculation of a benchmark dose in this report does not mean that regulation based on the underlying data is recommended, or even that the underlying data are suitable for regulatory decision-making. Values for this table were calculated using the probit model by CERHR using Environmental Protection Agency (EPA) Benchmark Dose Software version 1.3.2.

[b] Significantly different from control ($P < 0.05$), Fisher exact test by CERHR.

[c] Significant trend across doses, chi-squared test by CERHR.

From McClain et al., 2006 *(229)*.

inflammation was observed at ≥50 mg/kg/day. In female rats, the study authors reported histopathology alterations in ovaries and uterus/cervix at ≥50 mg/kg/day. [Although the authors claimed that squamous metaplasia of the cervix was increased at ≥50 mg/kg/day, the tables in the study indicate no such increase until 500 mg/kg/day.] Histopathological changes in vagina and mammary gland were observed at 500 mg/kg/day. The types of histopathology findings in female reproductive organs are outlined in **Table 65**. **[The study authors reported an increase in osteopetrosis in males and females at ≥50 mg/kg/day; however it appears that the increase at 50 mg/kg/day was observed only at 26 weeks in females (2/5 females of the 50 mg/kg/day group and 5/5 females of the 500 mg/kg/day group affected versus 0/5 controls affected).]** Extramedullary hemopoiesis **[incidence and severity not indicated]** was reported to occur in the spleen at all doses and was stated to be a compensatory response to decreased bone marrow resulting from bone thickening. Liver histopathology was observed in males and females at 500 mg/kg/day. Many of the histopathology observations observed at 52 weeks (i.e., effects in liver, bone, epididymides, prostate, ovaries, uterus, and vagina) were also observed at 26 weeks. Following the 8-week recovery period, osteopetrosis in females and epididymal vacuolation were the only persistent histopathological effects observed at the high dose.

Based on mild hepatic effects consisting of minimal bile duct proliferation and increased γ-glutamyl transferase activity, the study authors identified a NOAEL of 50 mg/kg/day. **[It is noted that study authors indicated an increase in ovarian atrophy and prostate inflammation at 50 mg/kg/day; it was not explained why the effects were not considered in the selection of a NOAEL.]**

McClain *et al.*, 2005 *(249)* examined the effects of subchronic and chronic genistein exposure on dogs. In a 4-week and a 52-week study, Beagle dogs were orally dosed with capsules containing genistein doses of 0, 50, 150, or 500 mg/kg/day. **[Funding support not stated, but author affiliations**

Table 65. Treatment-Related Histopathological Effects in Female Rats Given Genistein in Diet for 52 Weeks (McClain et al., 2006)

Effect	Animals Affected/Number Examined at each Genistein Dose, mg/kg/day			
	0	5	50	500
Fatty change in liver	6/20	13/19	17/20[a]	1/19
Bile duct proliferation in liver	0/20	3/19	0/20	6/19[a]
Hepatocellular hypertrophy	0/20	2/19	2/20	10/19[a]
Osteopetrosis	3/20	0/20	3/20	18/20[a]
Mammary gland secretion	1/20	1/1[b]	0/1[b]	6/20
Mammary gland proliferation	0/20	0/1[b]	0/1[b]	4/20
Ovary bursa dilatation	0/20	2/20	1/20	9/20[a]
Ovarian senile atrophy	11/20	14/20	17/20	18/20[a]
Cornual uterine dilation	1/20	1/20	2/20	3/20
Uterine hydrometra	0/20	0/20	0/20	7/20[a]
Uterine squamous hyperplasia	1/20	4/20	1/20	13/20[a]
Cervical squamous hyperplasia	1/20	0/20	1/20	5/20
Uterine gland squamous metaplasia	1/20	1/20	0/20	5/20
Vaginal mucification	4/20	7/20	4/19	14/19[a]
Vaginal cystic degeneration	3/20	3/20	1/19	10/19[a]
Vaginal epithelial hyperplasia	1/20	1/20	1/19	5/19

[a] Significantly different from control ($P < 0.05$), Fisher exact test by CERHR.
[b] **[It appears that the authors may have made an error in listing the total numbers of animals examined.]**
From McClain et al., 2006 *(229)*

include Nutritional Products and Hoffmann-La Roche, Ltd.] The purity of genistein was reported at 99.4–100%. Three dogs/sex/group were dosed in the 4-week study, and the authors stated that 4 dogs/sex/group were dosed in the 52-week study. **[Based on the number of dogs reportedly killed at different time intervals, it appears that the control and high-dose groups in the 52-week study contained 6 dogs/sex.]** Dogs were 5.5–6.5 months of age in the 4-week study and 5–6 months of age in the 52-week study. The dogs were fed a diet containing soybean meal as a protein source. The diet was analyzed and found to contain 77.1 ppm total genistein. Based on a daily feed intake of 300 g/dog, the study authors estimated that dogs would be exposed to an additional intake of total genistein of 23 mg/day or 2.3 mg/kg/day genistein for a 10 kg dog. However, this additional intake was considered to represent a small amount of the administered genistein doses of 50 to 500 mg/kg/day Body weight and feed intake were measured, and dogs were examined for viability, behavior, and clinical signs of toxicity. Ophthalmoscopic examinations were conducted and hematological, clinical chemistry, and urinalysis parameters were measured prior to testing, at the end of the 4-week study, and every 13 weeks in the 52-week study. In the 4-week study, all dogs were killed following the dosing periods. In the 52-week study, 2 dogs/sex/group were killed after 13 weeks of treatment and 2 dogs/sex/group were killed after 52 weeks of treatment. Two dogs/sex from the control and high-dose group were killed following a 4-week recovery period. At necropsy, organs were weighed and histopathological examinations were conducted. Toxicokinetic analyses were also conducted in the 4-week study and are discussed in **Section 2.1.2.2**. Statistical analyses included Dunnett and/or Steel tests.

In the 4-week study, the only clinical sign was a dose-related increase in pale feces or feces containing white particles. The authors speculated that white particles in feces may have been unabsorbed genistein, but they did not measure genistein levels in feces. Genistein had no effect on survival, body weight gain, feed intake, ophthalmoscopy findings, clinical chemistry measurements, urinalysis endpoints, or gross or histopathological alterations in organs. The only hematological finding was a slight decrease in fibrinogen levels in males of the 150 and 500 mg/kg/day group, but due to the small magnitude of effect in males and lack of effect in female dogs, the authors did not consider the finding to be treatment-related. Increases in absolute **[119%]** and relative **[133%]** uterine weights in high-dose females were the only organ weight effect observed. The uterine weight effects did not attain statistical significance.

In the 52-week study, genistein treatment had no effect on survival, body weight gain, feed intake, or ophthalmoscopy findings. Feces that were pale or contained white specks suspected to be unabsorbed genistein were observed, but no analyses were done to measure genistein levels in feces. Some statistically significant effects were observed for hematology and clinical chemistry parameters, but there were either no dose-response relationships or the findings were noted prior to exposure. Therefore, none of the hematology or clinical chemistry findings were considered treatment-related by study authors. No treatment-related effects were reported for urinalysis parameters **[data not shown by study authors]**. In male dogs, testis weight were markedly decreased in 2/2 dogs of the 500 mg/kg/day group following 13 weeks of treatment **[mean 75% decrease in relative weight, not statistically significant]** and in 1/4 dogs following 52 weeks of treatment **[mean 32% decrease in relative weight, $P<0.05$]**. Uterine weight was increased in the 500 mg/kg/day group following 13 weeks of exposure **[83% increase in relative weight, not statistically significant]** but not following 52 weeks of exposure. A slight reduction in ovary weight was described in the 150 and 500 mg/kg/day group following 13 weeks of treatment **[14% decrease in relative weight, not statistically significant]** and in the 500 mg/kg/day group following 52 weeks of treatment **[20% decrease in relative weight, not statistically significant]**. No other organ weight effects were considered treatment-related by study authors, and none of the organ weight changes persisted through the recovery period. **[The Expert Panel noted that changes in testicular, uterine, and ovarian weights at 13 versus 52 weeks of treatment suggest adaptation.]**

Gross organ observations in the 500 mg/kg/day group included decreased size of epididymides, testes, and/or prostate in 2 of 2 dogs at 13 weeks and in 1 of 4 dogs at 52 weeks. In the 150 mg/kg/day group, reduced size of epididymis, testis, and/or prostate was observed in 2/2 dogs at 13 weeks but was not observed at 52 weeks. Decreased ovarian sizes were observed in 1/2 animals of each dose group at 13 weeks. At 52 weeks, thickened mammary glands were observed in 1 control female, 2 females of the 150 mg/kg/day group, and 1 female in the 500 mg/kg/day group. None of the gross findings were observed following the recovery period. The authors noted some histopathological findings in males that they considered treatment related. No cases of testicular, epididymal, or prostatic atrophy were observed in control dogs or in dogs from the two lower dose groups. In the 500 mg/kg/day group, testicular atrophy was observed in 2/2 males at 13 weeks and 1/4 males at 52 weeks; epididymal atrophy was observed in 1/4 dogs at 52 weeks; and prostatic atrophy was observed in 2/4 dogs at 52 weeks. Testicular histopathology was characterized by small tubular diameter, reduced seminiferous epithelial height, occasional tubules containing only Sertoli cells, vacuolation of tubular epithelium, and presence of multinuclear giant cells. In epididymides, the epithelium was low in height and no spermatozoa were present. Prostatic acini were not well developed. No treatment-related histopathology changes were observed in male dogs following the recovery period. There

were no treatment-related histopathological findings in females. **[Changes in histopathology at 13 versus 52 weeks also suggest adaptation.]**

The study authors concluded that the transient effects of high genistein doses on the reproductive tract of dogs were functional and not considered to be adverse effects. Therefore the study authors identified a NOAEL of >500 mg/kg/day. **[Testicular atrophy and increased uterine weights were observed at that dose, but adaptation occurred.]**

2.2.2 Cardiovascular

Because estrogens have hypocholesterolemic properties and mortality rates for cardiovascular diseases are lower in populations consuming larger amounts of soy products, isoflavones have been suggested to be protective against cardiovascular disease. In 1999, after evaluating the literature, the U.S. FDA approved the following food-labeling health claim, or similar variant, for soy protein in the prevention of coronary heart disease: "Diets low in saturated fat and cholesterol that include 25 grams of soy protein a day may reduce the risk of heart disease. One serving of (name of food) provides (insert amount) grams of soy protein."

Similar petitions have been approved in 8 other countries including Japan in 1996, the United Kingdom in 2002, South Africa in 2002, the Philippines in 2004, Brazil in 2005, Indonesia in 2005, Korea in 2005, and Malaysia in 2006 (reviewed in *(250)*). The FDA approved the health claim based on evidence that soy foods as part of a diet low in saturated fat and cholesterol can lower total blood cholesterol and low density lipoprotein (LDL) levels. In 2003, the UK Committee on Toxicity reached a similar conclusion: "There is evidence from epidemiological studies and intervention trials that diets containing soy or soy protein isolates can have a hypocholesteromic effect in humans" *(3)*. Both the FDA and UK Committee on Toxicity concluded that there was no conclusive evidence that the hypocholesterolemic properties of soy products are due to isoflavones. **[On December 21, 2007, the FDA announced its intent to reevaluate the scientific evidence for the authorized unqualified health claim for soy protein and risk of CHD issued in 1999 *(251)*. As of August 2009, this issue is still under review at the FDA.]**

In August 2005, the American Heart Association (AHA) Nutrition Committee approved a science advisory statement on soy protein, isoflavones, and cardiovascular health *(252)* that concluded "Earlier research indicating that soy protein as compared with other proteins has clinically important favorable effects on LDL cholesterol and other CVD risk factors has not been confirmed by many studies reported during the past 10 years." This statement was based on a reevaluation of the 2000 conclusion reached by the AHA Nutrition Committee that "it is prudent to recommend including soy protein foods in a diet low in saturated fat and cholesterol." The AHA revisited its initial conclusion because of the number of well-controlled, randomized trials that have been conducted on soy protein or soy-derived isoflavones since approval of the 2000 statement.

The AHA separately considered studies that used soy protein with isoflavones (n=22), soy protein removed of isoflavones (n=7), and studies that compared soy protein with and without isoflavones or administered isoflavones in pill form (n=19). Based on the 22 randomized trials of soy protein with isoflavones, the AHA concluded that very large amounts of soy protein were required to achieve reductions of a few percentage points in LDL cholesterol concentrations, the average effect was 3%,

when soy protein replaced dairy protein or a mixture of animal proteins in the diet. The AHA considered the reduction in LDL to be quite small in relation to the amounts of soy protein used in the studies, more than half the daily protein intake, and noted that the data were mainly obtained from individuals with high cholesterol *(252; 253)*. The studies provided no evidence that soy protein consumption had beneficial effects on HDL cholesterol, triglycerides, lipoprotein(a), or blood pressure. The range of soy protein used in the 22 studies was 25 to 135 g/d and the range of isoflavones was 40 to 318 mg. The AHA also reviewed 7 trials with soy protein removed of isoflavones. These studies were designed specifically to distinguish between effects of the soy protein versus the isoflavones. These studies supported an effect of the protein, but not the isoflavones, on LDL cholesterol, an average decrease of 1 to 2% was calculated across all 7 studies. Studies that reported greater decreases in LDL cholesterol of 2 to 7% required daily consumption of large amounts of soy protein, 50 to 55g. From the 19 studies that allowed assessment of soy isoflavones, the AHA concluded the average effect on LDL cholesterol and other lipid risk factors was zero. The dose range of isoflavones used was 52 to 318 mg. While the AHA Nutrition Committee could not conclude that soy protein offered benefit compared to other proteins, the committee felt that "soy products should be beneficial to cardiovascular and overall health because of their high content of polyunsaturated fats, fiber, vitamins, and minerals and low content of saturated fat"*(252)*.

The cardiovascular effects of soy were also evaluated in a report prepared by Balk *et al.*, 2005 *(80)* for the Agency for Health Care Research and Quality (AHRQ) of the Department of Health and Human Services. While the 2005 AHA science advisory was based on the more recent studies, the report by Balk *et al.*, 2005 also considered the older literature. More than half of the studies included in the evaluation were considered by the technical panel to be of poor quality, i.e., use of single-cohorts, high or unequal drop-out rates between groups, inadequate accounting for important confounders, missing data, improper statistical analysis, etc. Overall, Balk *et al.*, 2005 concluded that soy may have a small effect on lipids. The median net change compared to controls in 61 studies that reported data on the effect of consuming soy products and total cholesterol was approximately ~5 mg/dL decrease (~2.5%). Results from meta-analysis related to LDL levels indicated a "…statistically, though not clinically, significant net decrease of 5 mg/dL (approximately 3%)." For triglycerides, a statistically significant net decrease of 8 mg/dL (~6%) was reported from the meta-analysis. No significant changes in HDL were found in the meta-analysis. Similarly, no effects were discernable for systolic or diastolic blood pressure, lipoprotein(a), C-reactive protein, homocystein, endothelial function, systemic arterial compliance, and oxidized LDL.

Cooke, 2006 *(254)* published a review on animal models used to investigate the health benefits of soy isoflavones. **[Funding support not stated but author is affiliated with Health Canada, Health Products and Food Directorate and the University of Ottawa.]** For cardiovascular effects, he reviewed studies in monkeys, rabbits, rats, mice, and hamsters. Cooke's overall conclusion was that the lipid-lowering effects of isoflavones have been established, but of less efficacy than thought previously.

2.2.3 Thyroid

Concerns about thyroid toxicity of genistein arose in the 1930s when goiters were observed in rats fed soybeans (reviewed in *(3; 255)*). In the 1950s and 1960s, cases of altered thyroid function, mostly goiter, were reported in infants fed soy formula. The problem was eliminated by adding more iodine to the formulas and replacing soy flour with soy protein isolate. Although the early reports of goiter in

infants fed soy formula have mostly ceased since manufactures began supplementing soy formula with iodine in 1959[9] there is still concern that use of soy formula in infants with congenital hypothyroidism may decrease the effectiveness of thyroid hormone replacement therapy, i.e., L-thyroxin *(255; 257)*. There are several reports of infants with congenital hypothyroidism on treatment with thyroid hormone medication who display persistent hypothyroidism despite thyroxin treatment when consuming soy food or who require a reduction in the amount of L-thyroxin required to maintain a euthyroid state upon withdrawal of soy formula (reviewed in *(257)*. The New Zealand Ministry of Health (MOH) recommends that clinicians treating infants with hypothyroidism who consume soy-based infant formula closely monitor the doses of thyroxin required to maintain a euthyroid state *(258)*. In addition, the MOH recommends that clinicians treating children for medical conditions who consume a soy-based infant formula be assessed for thyroid function if there are concerns for unsatisfactory growth and development. A 2003 report prepared by the Committee on Toxicity of Chemicals in Food, Consumer Products and the Environment *(3)* concluded that it is possible that the isoflavone content in soy-based infant formula may have the capacity to inhibit thyroid function in infants, although it was not established whether the concentrations of unconjugated isoflavones in these infants are sufficiently high to influence thyroid function.

In November 2006, the Senate Commission on Food Safety (SKLM) of the German Research Foundation adopted an opinion on isoflavones as phytoestrogens in food supplements and dietary foods for special medical purposes, e.g., alternative to hormone replacement therapy in post-menopausal women *(259)*. The focus of the SKLM evaluation was on the safety of these isoflavone products. Overall, they concluded that the safety of these preparations could not be derived from the traditional use of soy-based foods in Asian countries. With respect to thyroid effects, the SKLM reviewed epidemiological and clinical data and concluded that the consumption of soy foods is unlikely to have an adverse effect on the thyroid gland if iodine intake is sufficient. Similarly, Messina and Redmond, 2006 *(257)* reviewed 14 human trials and concluded that the literature provided little evidence that soy foods or isoflavones adversely affect thyroid function in people with normal thyroid function and sufficient iodine intake.

Balk *et al.*, 2005 *(80)* reviewed six randomized trials, including 4 with cross-over design, that reported the effect of soy on thyroid stimulating hormone (TSH) in post-menopausal women (4 studies), pre-menopausal women (1 study), or men (1 study). The technical panel that participated in the Balk *et al.*, 2005 review considered the studies to be of poor to moderate quality with mostly limited applicability to post-menopausal women. The duration of the studies ranged from 4 to 24 weeks. Only one study reported a statistically significant increase for TSH level in postmenopausal women. Although most of the studies in postmenopausal women showed a trend for TSH increase, this result was not consistent for all soy arms. The study in men reported a non-significant decrease in TSH for all the soy arms. Balk *et al.*, 2005 concluded that no overall effect of soy on TSH and thyroid function is clear. Limited evidence suggests a possible small increase in TSH level with consumption of soy products by post-menopausal women. However, the only trial with men reported a decrease in TSH level with soy consumption. These differences were small and not expected to have a clinical effect on thyroid function.

A 1991 study of Japanese men is often cited as evidence that soy isoflavones can impact thyroid function in healthy subjects. In this study, Japanese men fed 30 g soybeans/day for 1 or 3 months

[9] In 1998, the New Zealand Ministry of Health noted one case report published by Labib *et al.*, *[256]* on thyroid abnormalities associated with soy-based infant formula since iodine supplementation.

reported signs of thyroid toxicity including increased thyroid-stimulating hormone levels (TSH), decreased thyroxine, and diffuse goiter (Ishizuki *et al.*, 1991 reviewed in *(3; 255)*). Symptoms of constipation, fatigue, and lethargy were observed in about half the subjects treated for 3 months. Recovery was observed following cessation of the soybean diet. Based on levels of isoflavones in Japanese soybeans, Fitzpatrick, 2000 *(255)* estimated intakes of 23 mg/day genistein (0.33 mg/kg for a 70-kg adult) and 10 mg/day daidzein (0.14 mg/kg for a 70-kg adult). Other studies in humans ingesting up to 132 mg/day isoflavones, through soy consumption in most cases, reported small and variable effects on thyroid hormone levels that the study authors generally did not consider clinically significant (reviewed in *(3)*). Thus, the Ishizuki *et al.*, 1991 study has been considered an outlier in the literature on soy consumption and thyroid function in healthy adults. The review by Messina and Redmond, 2006 *(257)* noted a number of experimental weaknesses in the Ishizuki *et al.*, 1991 study. Specifically, the study did not include a control group and did not provide an adequate description of the soy product fed to participants. In addition, Messina and Redmond considered it biologically implausible that the relatively small amounts of soy protein and isoflavones used in this study could result in marked antithyroid and goitrogenic effects in a population that regularly consumes soy but does not have a high incidence of goiter.

A number of studies in humans have been published since the previous CERHR evaluations of genistein and soy formula that include an assessment of the thyroid. Studies involving infants or children are presented in chapter 3 and studies in adults are briefly described here. Dillingham *et al.*, 2007 *(260)* reported no effects on serum T3, free T3, total T4, free T4, TSH, or TBG in 35 healthy young men (aged ≥20 years) who consumed either a milk protein, low-isoflavone protein, or high-isoflavone protein isolate for a period of ~ 2 months. Hampl *et al.*, 2008 *(261)* assessed the short-term effects on thyroid hormones resulting from consumption of unprocessed boiled soybean consumption over a 7-day period (2 g/kg body weight/day) in male (n=32) and female (n=54) university students. In men, but not women, consumption of the soybeans was associated with a significant increase in TSH. The authors also reported several significant associations between serum levels of unconjugated daidzein and thyroid hormones but no significant associations with serum levels of unconjugated genistein. Significant associations were reported between serum levels of unconjugated daidzein basal levels of daidzein and thyrotropin in men, daidzein and antithyroglobulin at the end of the 7-day period in men, and between daidzein and free thyroxin at the end of the soy consumption period in women. Overall, the authors considered these effects modest and transitory.

While the SKLM and other reviews cited above generally conclude that the consumption of soy foods is unlikely to have an adverse effect on the thyroid gland if iodine intake is sufficient, the SKLM report also concluded that data from animal experiments suggested that high intake of isoflavones may cause adverse effects in situations where there is an iodine deficiency or hypoactivity of the thyroid gland. For example, evidence of thyroid toxicity (e.g., increases in thyroid weight and thyroid-releasing hormone level, decreased thyroxin level, and histological changes) was observed in iodine-deficient rats fed soybeans, but there was no effect when rats were fed soy providing genistein concentrations of 60 mg/kg diet or isoflavones at ≤2000 mg/kg diet (reviewed in *(3)*). One study of iodine-deficient female rats fed soybeans reported an increase in thyroid carcinoma (Kimura *et al.*, 1976 as reviewed by the UK Committee on Toxicity, 2003 *(3)*) , but no evidence of carcinogenicity was observed in a second study examining effects of genistein intake (≤250 mg/kg diet) in rats (Son *et al.*, 2000a and 2000b as reviewed by the UK Committee on Toxicity, 2003 *(3)*).

Because the incidence of subclinical hypothyroidism increases with age, occurring in ~ 10% of women over the age of 55, the SKLM considered post-menopausal women taking isoflavone-containing food supplements at risk for potential side-effects on thyroid function. Other potentially at risk populations identified by the SKLM included people born without a functioning thyroid gland or those who have their thyroid glands partially or completely removed due to the presence of a thyroid tumor or a Morbus Basedow-type hyperthyroidism (also referred to as Grave's disease). Conclusions presented in reviews by Fitzpatrick, 2000 *(255)*, Messina and Redmond, 2006 *(257)*, Doerge and Chang, 2002 *(262)* and the UK Committee on Toxicity *(3)* also recognize that individuals with hypothyroidism and/or inadequate iodine intake may be more susceptible to thyroid effects following soy intake or that soy may interfere with medications used to treat thyroid hormone conditions.

Several targets of isoflavones in the thyroid hormone system have been identified. A number of *in vitro* and *in vivo* studies show that soy isoflavones can act as competitive substrates for thyroid peroxidase (TPO), an enzyme found in thyroid follicle cells that catalyzes two reactions required for thyroid hormone synthesis (reviewed in Doerge and Chang, 2002 *(262)*). *In vitro* studies show that genistein, daidzein, and genistin (to a much lower extent) have the ability to inhibit TPO-catalyzed reactions. Genistein in combination with hydrogen peroxide inhibited activity of TPO obtained from cows, pigs, rats, and humans (reviewed in *(13)*). In the absence of iodine, genistein can cause irreversible loss of enzyme activity. Doerge and Chang, 2002 suggested the loss of activity could occur through genistein interactions with reactive TPO intermediates and the subsequent formation of reactive isoflavone radicals which combine to create a covalently modified form of the enzyme. In the presence of iodine, they act as competitive substrates for tyrosine iodination; thus iodine can mitigate genistein-mediated loss of TPO activity. Fitzgerald, 2000 reviewed this literature and noted that genistein appears to be a more potent inhibitor of TPO than the anti-thyroid drugs methimazole and 6-propylthiouracil *(255)*.

The predicted effects *in vivo* from TPO inhibition would be reduced concentrations of thyroid hormone and increased production of TSH. Studies conducted at the National Center for Toxicological Research (NCTR) involving administration of genistein or soy to rats show that inhibition of TPO activity can also be observed *in vivo,* including at administered dose levels of genistein that result in blood levels of total genistein similar to those observed in various human populations (Chang and Doerge, 2000 as reviewed in Doerge and Chang, 2002 *(262)*). However, the predicted decreases in T3/T4 and increased TSH were not observed in either the genistein or soy-fed rats. In addition, no differences in thyroid weight or histopathology were observed at the highest dose tested in a separate study with the same experimental design. These findings, coupled with studies by other investigators using iodine-deficient rodent models, suggest that impaired thyroid function, e.g., iodine deficiency, is necessary for soy to exert anti-thyroid effects in rats *in vivo*. A study by Ikeda *et al.*, 2000 (reviewed in *(262)*) reported that whole soy, as opposed to individual isoflavones, was required to produce a hypothyroid state in rats under conditions of iodine insufficiency.

In addition to TPO inhibition, other mechanistic targets have been identified that may contribute to understanding the reported effects of soy on thyroid function (reviewed in *(259)*). Isoflavones can inhibit sulfotransferases which are involved in the inactivation and elimination of thyroid hormones as well as the reutilization of iodine in the thyroid gland (Ebmeier and Anderson, 2004 as reviewed in Eisenbrand 2007 *(259)*). In addition, genistein and other isoflavones have been shown *in vitro* to be inhibitors of the binding of T3 and T4 to the thyroid-hormone transport protein transthyretin (TTR) (*(263; 264)* reviewed in *(259)*)

. Radovic *et al.*, 2006 *(264)* reported that genistein was a strong competitor for TTR, showing a binding affinity comparable to that of labeled T4. Reviews by Xiao *et al.*, 2006 and 2008 *(265; 266)* summarized research from their lab found that rats dosed with soy protein isolate, but not isolated isoflavones, had significantly increased liver content of thyroid receptor b1 protein, a key regulator of lipid metabolism. Other tissues and other isoforms of the thyroid receptor were not altered, suggesting the effects are tissue- and isoforms-specific. There is also an *in vitro* study reporting that genistein can induce proliferation in thyroid cancer cells through G protein-coupled receptor (GPR30) and mitogen-activated protein kinase (MAPK) pathways *(267)*. There is also data indicating that genistein can affect thyroid hormone function in non-mammalian experimental models. In amphibians, tadpole tail reabsorption is controlled by thyroid hormones and Ji *et al.*, 2007 *(268)* showed that genistein can inhibit T3-induced tail regression in organ culture. Because tyrosine phosphorylation is important in this metamorphic event, the authors conducted experiments to evaluate phosphorylation signaling pathways and concluded that genistein could be causing the inhibitory effect on tail reabsorption due to its known activity as a tyrosine kinase inhibitor.

2.2.4 Allergy and Immunology

The prevalence of soy allergy in infants and children is estimated to be 0.3 to 0.4% and it is commonly cited as one of the eight most common contributors to IgE-mediated food allergies in children, along with cow's milk, hen's eggs, peanuts, tree nuts (and seeds), wheat, fish, and shellfish *(269; 270)*. A recent review by Zuidmeer *et al.*, 2008 *(271)* similarly reported soy allergy prevalence below 1% regardless of method used to assess or age group, although higher prevalence estimates of ~3% were reported in a number of Swedish papers included in the review. Allergic reactions to soy are most commonly manifest as hives, atopic dermatitis, and gastrointestinal symptoms although there are rare reports of severe anaphylaxis in children. Soy is also a main causative allergen in infants with food protein-induced gastrointestinal syndromes (FPIES), although infants with FPIES commonly have multiple food allergies *(270)*. Young children who have hypersensitivity to a number of food products, including soy, may be at increased risk of developing atopic dermatitis *(272)*. In contrast to allergies to peanuts and shellfish, soy allergy generally does not persist and many children develop tolerance by shecol age and ~ 85% of children with IgE-mediated allergies to soy will outgrow them by the age of three *(269; 270)*. One study in adults with a history of soy allergy reported wide variability in the amount of soy, 10 mg to 50 g, required to induce "subjective" indications of allergic response *(273)*.

The American Academy of Pediatricians (AAP) does not recommend soy during the first year of life as a strategy to prevent the development of allergies to other foods in infants at high risk for developing food allergies *(42)*. After reviewing the literature, the AAP and others have concluded that use of soy formula is not effective at preventing the development of allergies in later infancy and childhood in infants at high risk of developing food allergies when compared to cow's milk-based formula *(42; 274)*. In addition, while most children with IgE-mediated cow's milk allergy tolerate soy, there is a ~ 10% to 14% crossover rate with soy allergy *(42)*. The crossover rate of allergenicity may be higher in cases of non-IgE-mediated cow's milk allergy *(35)*. However, approximately 98% of children with cow's milk allergy are able to tolerate an extensively hydrolyzed cow milk formula *(269)*. These types of observations led the AAP to recommend hydrolyzed or extensively hydrolyzed formula instead of a soy-based formula in infants with cow's milk allergy.

Similarly, the ESPGHAN Committee on Nutrition expressed a preference for the use of an infant formula based on extensively hydrolyzed proteins (or amino acid preparations if hydrolysates are

not tolerated) over a soy protein formula in the treatment of cow's milk protein allergy *(36)*. The Committee concluded that soy protein formula should not be used in infants with food allergy during the first 6 months of life. After that point, if soy protein formula was being considered based on factors such as lower cost and better acceptance, tolerance to soy protein should first be established by clinical challenge. The Committee concluded that soy protein formulae did not have a protective role in the prevention of allergic diseases.

In 2008, an Australian consensus panel developed an opinion on guidelines for use of infant formulas to treat cow's milk protein allergy *(275)*. **[Meeting teleconferences and face-to-face meetings were funded by SHS/Nutricia, a medical nutrition company that produces infant formulas, including amino-acid based, hypoallergenic formulas. While none of the authors of the consensus panel were compensated in exchange for their participation, a number of the authors had relationships with Nutricia or other producers of infant formula that ranged from speaker fees, honoraria, membership on advisory committees, or support for research.]** Their overall conclusions were:

1. Three types of infant formula (soy, extensively hydrolysed and amino acid) may be appropriate for treating cow's milk protein allergy.
2. Selection of a formula depends on the allergy syndrome to be treated.
3. Extensively hydrolysed formula is recommended as first choice for infants under 6 months of age for treating immediate cow's milk allergy (non-anaphylactic), food protein-induced enterocolitis syndrome, atopic eczema, gastrointestinal symptoms and food protein-induced proctocolitis.
4. Soy formula is recommended as first choice for infants over 6 months of age with immediate food reactions, and for those with gastrointestinal symptoms or atopic dermatitis in the absence of failure to thrive.
5. Amino acid formula is recommended as first choice in anaphylaxis and eosinophilic oesophagitis.
6. If treatment with the initial formula is not successful, use of an alternative formula is recommended.

Estrogens are known to be important in the normal development of the immune system as well as implicated in a variety of immune disorders *(276; 277)*). A large number of studies have reported that genistein has inhibitory effects on immune cell function *in vitro* (reviewed in *(277; 278)*). These findings, especially those occurring at high concentrations, may be difficult to extrapolate to lower concentration effects because genistein is known to inhibit tyrosine kinases, enzymes involved in many facets of immunological signaling, at supraphysiological concentrations, generally on the order of ≥100 µmol/L. *In vivo* findings in laboratory animals exposed to genistein as adults include altered thymic size, thymocyte apoptosis, decreased CD4+ thymocytes or no change in thymic cellularity, decreased humoral and cell-mediated immunity, prolonged cardiac allograft survival, decreased production of the cytokine IFN-γ, lower IFN-γ response to bacterial infection, increased host tumor resistance, increased IL-2 stimulated NK cell activity, increased basal splenocyte proliferation, and decreased Con-A activated splenocyte proliferation (based on 5 studies in adult animals reviewed in *(277)*).

2.2.5 Menopausal Symptoms

Vasomotor symptoms are a common complaint for menopausal women. Eighty per cent of all menopausal women will have hot flushes and night sweats, and of these 9% will have severe symptoms impacting their quality of life *(279)*. For most women, vasomotor symptoms spontaneously resolve in

3 - 5 years. The frequency of these symptoms can vary by culture and estimates of hot flash incidence are far lower in Asian countries compared to Western countries. One estimate is that between 70–80 % of women in the U.S. and Europe report having hot flashes, compared to 10–20 % of women in Japan, Singapore and China (reviewed in *(280)*). Suggested explanations for the differences in hot flash incidence include variation in isoflavone intake from traditional diets and/or a cultural reluctance of Asian women to discuss the topic.

Balk *et al.*, 2005 *(80)* reviewed human studies that assessed the effects of soy and/or soy isoflavones on menopausal symptoms. The majority of these trials studied vasomotor symptoms such as hot flashes and night sweats. However, a number of the studies also included assessment of depression, anxiety, insomnia, palpitations, and loss of libido. Conducting a meta-analysis on the studies was considered inappropriate because of the wide variety of indices used to measure vasomotor symptoms. In general, Balk *et al.*, 2005 concluded that the literature on the effects of soy protein and/or its isoflavones on menopausal symptoms were inconsistent and difficult to interpret. For example, placebo effects were observed in all the human trials such that decreases in hot flash indices were reported in both the treatment and control groups. Approximately a third of the studies in post-menopausal women found no effect on hot flashes or a worsening effect compared to control. The remaining two-thirds showed either statistically non-significant or significant decreases in hot flashes. Indication for a beneficial effect was stronger in the randomized trials with isoflavone supplements, reductions in hot flash frequency of 7 to 40%, but most of these studies were considered of poor quality due to high dropout rates that were often uneven between soy treatment and control groups. The overall conclusion presented in Balk *et al.*, 2005 was that "soy isoflavone supplements might reduce hot flashes in symptomatic post-menopausal women, compared to placebo."

Four studies evaluating soy consumption and symptoms of menopause in peri-menopausal women or those receiving breast cancer therapy were evaluated by Balk *et al.*, 2005. These studies did not provide evidence that soy and/or its isoflavones provided a reduction in vasomotor symptoms compared to control, the changes in symptoms reported in these studies ranged from -77% to +23%.

The American Heart Association also considered the literature on soy isoflavones and improvement of menopausal vasomotor symptoms in its 2006 science advisory statement *(252)*. Less than a third of the studies reviewed reported modest improvements in hot flashes with the longest studies showing no benefit of isoflavone treatment. Reductions in hot flashes on the order of 40 to 60% were reported in the placebo or control groups in the reviewed studies. The overall AHA conclusion was that "…it seems unlikely that soy isoflavones have enough estrogenic activity to have an important impact on vasomotor symptoms of estrogen deficiency in peripausal women." The UK Committee on Toxicity report from 2003 similarly concluded that the literature on soy-based products or isoflavones to relieve menopausal symptoms was inconclusive and equivocal as the beneficial results were often not statistically significant and strong placebo responses were noted *(3)*. Of the 12 studies reviewed by the Committee, half reported that soy diets or isoflavone supplementation reduced the frequency of hot flashes, and the other half reported no effect on hot flashes.

Several other reviews suggest that the literature evaluations that do not discriminate between the identities of individual isoflavones in the study product may be misleading. In 2006, staff of the Archer Daniels Midland Company, a major producer of isoflavone supplement products, identified and reviewed 11

studies where the isoflavone-containing supplement was well characterized *(281)*. All 11 studies contained similar total isoflavone levels but varied in genistein content (calculated as aglycone equivalents). All 5 studies that used study products containing > 15 mg genistein reported statistically significant decreases in hot flash symptoms whereas only one of 6 studies that used a study product containing less than 15 mg genistein reported a significant decrease in hot flash. This evaluation led the authors to conclude that reduction in hot flashes appear to be related to genistein dose and not total isoflavone content.

The consensus view on soybean phytoestrogens in clinical interventions in post-menopausal women was presented Cassidy *et al.*, 2006 *(145)*. **[There was no declaration of conflict of interests by the author in the consensus report. One author was affiliated with Nestle, a manufacturer of soy infant formula and other soy products.]** The consensus panel concluded that there was limited evidence that soy protein isolate (SPI), soybean foods, or red-clover extract are effective in reducing symptoms of menopause. The strongest indication for an effect was based on reports of reductions in hot flashes in 6 of 8 studies that used isolated isoflavones or purified genistein. Based on these studies, Cassidy *et al.*, 2006 concluded that soy bean isoflavones may reduce hot flashes although they appear less effective than reductions observed with hormone-replacement therapy and similar to other non-hormonal pharmacological therapies.

2.2.6 Bone Density

Declines in bone-mineral density accelerate during late perimenopause and the first years following menopause with reported yearly declines of 1 to 2.3%, with larger rates of decline reported for women in the lower tertile of body weight *(282)*. At this pace over a 5-year span, the average woman's bone mineral density would decline in the spine and hip to amounts that are associated with 50–100% higher fracture rates. Because the loss of bone mineral density may be the result of reduced estrogen, a number large number of studies in experimental animals and humans have assessed whether soy protein or soy isoflavones have beneficial effects on bone health *(3; 145)*.

Liu *et al.*, 2009 *(283)*, supported by Sun Yat-sen University, Guangzhou, China, conducted a meta-analysis randomized clinical trials that were published from between January 1990 and March 2008 and that included soy isoflavone supplementation in women for at least one year. The main outcomes were bone mineral density (BMD) changes from baseline at the lumbar spine, total hip, and femoral neck. Ten studies containing 896 women were identified. A major limitation of the meta-analysis was the large differences between studies on soy isoflavone effects and BMD at the lumbar spine and femoral neck which may be the result of differences in both the doses of soy isoflavones used and study quality. The results of the meta-analysis showed that ingestion of an average dose of 87 mg soy isoflavones for at least one year did not significantly affect BMD with mean differences of 0.4% at the lumbar spine, -0.3% at the femoral neck and 0.2% at the total hip. The authors reported similar findings when analyses were based on isoflavone source (soy protein versus isoflavone extract), ethnicity (Asian versus Western). Larger doses of isoflavones (≥80 mg/d) appeared to have weak beneficial effects on spine BMD, P=0.08. Overall, the authors concluded that soy isoflavones were unlikely to have beneficial effects on BMC at the lumbar spine and hip in women.

Balk *et al.*, 2005 *(80)* reviewed 31 human studies that included assessment of bone health, including bone mineral density (BMD), bone formation markers (bone specific alkaline phosphatase and osteocalcin) or bone resorption biomarkers (urinary hydroxyproline, urinary cross-linked N-telopeptide, urinary pyridinoline, and urinary deoxypyridinoline). Overall, the limited availability of long-term randomized

trials and wide variety of soy interventions used made drawing a reliable overall conclusion difficult. In general, no significant effects on bone mineral density or bone formation markers were reported when compared to control groups. No consistent effects were evident between soy protein with isoflavones and soy protein isolated of isoflavones. For bone resorption biomarkers, non-significant or inconsistent effects were reported. Although a number of studies reported reductions in two markers of bone resorption, urinary pyridinoline and deoxypyridinoline, other markers of bone resorption were not affected and the effects were not consistent across studies. There was also no indication of a dose effect for soy protein or soy isoflavones for these measures. Balk *et al.*, 2005 cited a study that used 54 mg tablets of purified genistein as the only studied included in the evaluation that reported consistent effects of bone measures, i.e., increased bone mineral denisity, increased markers of bone formation, and reduction on markers of bone resorption. Balk *et al.*, 2005 noted that the women in this study had a baseline measure of bone mineral density in the femoral neck that was lower than ~50% of the general population. In addition, this study used a purified preparation of genistein whereas most of the other studies used soy protein or soy isoflavone extracts.

The American Heart Association also reviewed a number of clinical trials that included assessment of bone loss *(252)*. They noted that the existing clinical trials generally had insufficient duration and size and had variable results. The results from studies with soy isoflavones were inconsistent with some showing beneficial effects such as reductions in bone loss and others not showing beneficial effects. The studies that showed beneficial effects were often internally inconsistent. For example, showing benefit in the spine but not the hip or improved bone mineral content without a change in bone mineral density. The longest study in cynamolgus monkeys, 3-years, showed the increased bone mineral density and content with estrogen replacement therapy but no effect on slowing bone loss with soy isoflavones.

The consensus opinion presented in Cassidy *et al.*, 2006 *(145)* was for a suggestion, but no conclusive evidence, that isoflavones had beneficial effects on bone health. Six human studies met the inclusion criteria of the working group. Two of the 3 studies that used soy bean isoflavone extract or pure genistein reported "suggestive" effects on bone mineral density while 1 of the 3 studies of soy protein isolate showed an effect. They concluded that further long-term studies of more than 1-year duration would help clarify the effects on bone.

Epidemiologic studies reviewed by the UK Committee on Toxicity *(3)* reported higher bone mass in populations consuming more soy products. Clinical data in humans are limited to relatively short-term studies and intervention trials that report inconsistent outcomes. A small number (~6) of short-term intervention studies in humans consistently demonstrated small but statistically significant soy-associated increases in bone mineral density in the lumbar spine. Other studies examining effects at other sites produced equivocal findings, with some reporting beneficial effects on bone mineral density and others reporting no effect. The UK Committee on Toxicity stated that long-term studies are needed before conclusions can be made about the effectiveness of phytoestrogens in improving bone health.

Experimental animal studies reviewed by the UK Committee on Toxicity *(3)* consistently demonstrated that soy isoflavones prevented bone loss in ovariectomized rodents. **[The Expert Panel noted that many studies of bone health and genistein were performed with genistein given immediately following ovariectomy. In contrast, women are often post-menopausal for a period of 2 years prior to genistein intake, which may result in loss of estrogen receptor (ER).]** Cooke, 2006 *(254)*

published a review on animal models used to investigate the health benefits of soy isoflavones and concluded that studies in monkeys, rats, and mice do not show a consistent benefit of isoflavones in preventing osteoporosis. However, Cooke noted the studies typically involved ovariectomized animals which may not be good models for menopausal women because ovariectomy results in a sudden drop in estradiol levels whereas hormonal changes are more gradual during menopause.

2.2.7 Reproductive Hormones and Function

Testosterone

Balk *et al.*, 2005 *(80)* reviewed 47 publications that included endocrine measurements. Five studies, 3 randomized controlled trials and 2 cohort studies, included assessment of testosterone in healthy men before and after consumption of soy protein (as isolate or in food) or pure soy isoflavones. Four of the 5 studies reported non-significant decreases in testosterone levels, a pattern which was interpreted as limited evidence to suggest a possible trend to lower risk of prostate cancer with consumption of soy products by men. The studies were considered generally too small and of poor quality to make meaningful conclusions.

Follicle Stimulating Hormone (FSH)

Balk *et al.*, 2005 *(80)* reviewed two studies, 1 randomized controlled trial and 1 cohort study, that investigated the effect of isolated soy protein or pure soy isoflavones on FSH levels among males. Overall, evidence was conflicting on the effect on FSH of soy product consumption by men. Six studies, 2 randomized controlled trials and 4 cohort studies, were reviewed that investigated the effect of consumption of soy protein or pure soy isoflavones on FSH levels in premenopausal women. A larger number of studies were available for post-menopausal women. The sixteen studies reviewed by Balk *et al.*, 2005 *(80)*, 14 randomized controlled trials and 2 cohort studies, presented conflicting evidence on the effect of soy consumption on FSH levels among post-menopausal women. All the studies that included assessment of FSH were considered to be of generally poor to moderate quality and of limited applicability. In addition, there was insufficient evidence to compare effect based on differences in types or doses of soy products.

Estradiol

Balk *et al.*, 2005 *(80)* reviewed a total of 26 studies on estradiol and soy and conducted separate analyses for pre- and post-menopausal women. Twelve studies on estradiol levels in 434 pre-menopausal women. The studies, six randomized controlled trials, 1 non-randomized controlled trial, and 5 cohort studies, were generally considered to be of poor quality and limited applicability. The overall effect of soy on estradiol levels was not consistent. Most of the studies showed a trend for soy in reducing estradiol, although they failed to demonstrate a significant effect. Fourteen studies, 10 randomized controlled trials and 4 cohort studies, investigated the effect of isolated soy protein or pure soy isoflavones on E2 levels among post-menopausal women. The studies were judged by the technical panel to be of generally poor to moderate quality and limited to moderate applicability. As was the case for pre-menopausal women, the evidence in post-menopausal women was considered conflicting. There was insufficient evidence on the different types or doses of soy products to compare their relative effectiveness.

In vitro studies suggest that genistein can inhibit the enzymes aromatase (involved in estrogen production), 5α-reductase (involved in testosterone metabolism), and 17β-hydroxysteroid dehydrogenase Type I (involved in the biosynthetic pathway from cholesterol to the sex steroids) (reviewed in *(3; 108)*). However, the effects were not consistently reproduced in whole-animal studies.

Whitten and Patisaul *(108)* noted that two studies in male rats fed phytoestrogens found no effect on brain aromatase activity, while one of the studies reported unspecified changes in 5α-reductase activity in the amygdala and preoptic area. It has also been reported that genistein inhibits CYP1A1, an enzyme that degrades 17β-estradiol, in a mouse hepatoma cell culture *(284)*.

It has also been suggested that isoflavones can alter circulating levels of estrogen and testosterone through their actions on sex hormone-binding globulin, a plasma protein that limits the free concentrations available for cell uptake and implementation of biological effects *(3)*. One theory is that isoflavones can inhibit binding of estrogens or androgens to sex hormone-binding globulin, thus increasing circulating levels of free hormones. The other theory is that isoflavones can increase synthesis of sex hormone-binding globulin, thus reducing circulating levels of free estrogens and androgens. Whitten and Patisaul *(108)* noted that studies examining binding affinities of phytoestrogens with sex hormone-binding globulin have produced inconsistent results. The UK Committee on Toxicity *(3)* noted that genistein binds weakly to sex hormone-binding globulin and concluded that phytoestrogens are unlikely to prevent binding of estrogen or androgens at genistein levels found in blood (<5 μM **[<1351 μg/L]**). *In vitro* studies demonstrated that genistein (≥5 μM **[<1351 μg/L]**) increases synthesis of sex hormone-binding globulin *(3)*. However, studies in humans given isoflavones reported inconsistent effects on sex hormone-binding globulin synthesis *(3; 108)*. One study reviewed by Kurzer *(285)* suggested that effects on estrogens and androgens mediated by sex hormone-binding globulin may be related to the ability to produce the daidzein metabolite equol, which is present in 30–40% of individuals. In that study, reduced androgen and estrogen levels and increased sex hormone-binding globulin concentrations were observed in premenopausal women who excreted equol.

A study released subsequent to the reviews examined the effects of genistein and other isoflavones on *in vitro* glucuronidation of 17β-estradiol *(286)*. Microsomes were obtained from the liver of a 63-year-old male and incubated with 17β-estradiol alone or together with genistein, daidzein, or glycitein. Formation of estradiol 3-glucuronide (catalyzed by UGT1A1) and estradiol 17-glucuronide (catalyzed by UGT2B7) were measured by HPLC. Genistein inhibited formation of estradiol 3-glucuronide **[by ~80%]** but had no effect on formation of estradiol 17-glucuronide. In contrast, daidzein stimulated production of estradiol 3-glucuronide by ~50% but inhibited formation of estradiol 17-glucuronide by ~15%. The effects of glycitein were similar to those of daidzein. Results were confirmed using genetically engineered Sf-9 insect cells expressing *UGT1A1*, which is involved in the formation of the 3-glucuronide. **[Concentrations of isoflavones and 17β-estradiol used in the studies were not reported, which makes interpretation of data difficult, as shown by an examination of dose-response relationships for daidzein.]** At a concentration of 25 μM 17β-estradiol, maximum stimulation of estradiol 3-glucuronide production was observed with daidzein concentrations of 5–50 μM. Daidzein concentrations exceeding 50 μM inhibited formation of the 3-glucuronide. The study authors concluded that daidzein may lower 17β-estradiol levels in tissues expressing *UGT1A1* **[The Expert Panel notes that a concentration of 25 μM 17β-estradiol is considered high.]**

Menstrual cycle length

Balk *et al.*, 2005 *(80)* reviewed a total of 11 trials in 10 publications evaluating effect of soy on menstrual cycle length in pre-menopausal women. Despite use of a wide range of soy interventions and comparisons in the trial which complicated synthesis, 10 of the 11 trials did not report a significant change. The overall conclusion was that no effect was observed on menstrual cycle length in the soy studies.

2.2.8 Cognition and Diabetes

Cognition

While the prevalence rates for all-cause dementias and vascular dementia are similar in Japan, China, and North America, the prevalence of Alzheimer's disease appears to be ~2.5-fold higher in Western countries compared to Japan and China *(287)*. This type of observation, along with the estrogenic activity of certain isoflavones, has led to questions on whether soy isoflavones may have protective effects on cognition.

Balk *et al.*, 2005 *(80)* reviewed 4 studies that examined the effects of soy products on cognitive function. The studies included three randomized controlled trial studies involving a total of 261 post-menopausal women. Two of these 3 studies in post-menopausal women used isoflavone supplements and were considered to be of medium quality; the other used an isolated soy protein supplement and was judged of high quality. The fourth study on 27 male and female college students was a randomized controlled trial of low quality. Balk *et al.*, 2005 concluded that the few available studies were too heterogeneous to draw an overall conclusion regarding the effects of soy protein and/or its isoflavones on cognitive function. The only long-term and high quality study reported no significant differences on cognitive function between the groups of post-menopausal women consuming soy protein with isoflavones or milk protein. Similarly, the consensus panel opinion presented in Cassidy *et al.*, 2006 *(145)* concluded that the available evidence does not support a beneficial effect from soy bean products or isoflavones on cognitive function in post-menopausal women.

Zhao and Brinton, 2007 *(287)*, supported by the Alzheimer's Association and the Kenneth T and Eileen L Norris Foundation, reviewed eight intervention studies that looked at the relationship between soy isoflavones and cognition in humans. Four of the seven studies in post-menopausal women reportedly demonstrated beneficial effects on verbal memory and frontal lobe function while the other three did not. In addition, the study of 27 college students considered by Balk *et al.*, 2005 was considered to show beneficial effects on certain cognitive functions such as verbal and non-verbal short-term memory, long-term memory, and "metal flexibilities." Overall, Zhao and Britton concluded that while a subset of studies report beneficial effects, definitive conclusions cannot be reached because the studies are inconsistent.

Diabetes

The consensus statement presented in Cassidy *et al.*, 2006 *(145)* concluded that soybean consumption may reduce the risk of diabetes. A total of 10 studies were identified that examined the effect of phytoestrogens on diabetes in post-menopausal women, although only 4 met the inclusion criteria required for detailed review. The overall conclusion that the available evidence is suggestive of an effect on diabetes was based on two studies that reported beneficial effects on glycemic control following ingestion of a soybean protein plus isoflavone mixture or a soybean replacement meal. Two other studies included in the detailed review did not report significant effects on glycemic control. Cassidy *et al.*, 2006 noted that direct comparison of the studies is difficult because of differences in the glycemic endpoints assessed, isoflavone preparation and composition of study population, i.e., men, women, pre-menopausal or post-menopausal women. Similar conclusions were reached in a review by Cederroth and Nef, 2009 *(288)*.

Balk *et al.*, 2005 *(80)* reviewed six studies that reported the effects of soy intervention on fasting blood glucose in non-diabetic populations One other study did not present data but reported only no significant effect on fasting blood glucose. All of the studies found no significant changes in fasting blood glucose (or glucose tolerance test) with soy intervention.

2.2.9 Estrogenicity

Estrogenicity is a property that is defined based on a biological response. The term "estrogen" is derived from a Greek root referring to the induction of sexual behavior. Historically, estrogenicity was defined based on the ability to induce uterine growth in immature or castrated rodents. The uterine hypertrophy assay is still in use, although additional assays have been developed to probe interactions of the test chemical and ERs. *In vitro* estrogenicity assays may include ER-binding assays, recombinant mammalian and yeast cell transcription assays, or cell proliferation *(3; 108)*. ER-binding assays indicate the test compound's affinity for the receptor compared to a reference compound such as 17β-estradiol but do not demonstrate if the test compound will act as an agonist or antagonist. Potential agonistic or antagonistic ability of compounds may be identified through using reporter gene expression or measuring cell proliferation responses, although cell proliferation is not necessarily specific to estrogenic effects or considered sufficient to demonstrate agonism or antagonism. In order to demonstrate agonism or antagonism, specific changes in target gene expression should be demonstrated and use of a knockout/knockdown model should be used to further demonstrate specificity. Mammalian and yeast cells have been engineered to express ERα/ERβ and a reporter gene controlled by an estrogen response element. The reporter gene usually codes for an enzyme that can be measured through quantification of activity or through measurement of transcript or protein levels. In estrogen-dependent cells, phytoestrogens were observed to both stimulate and inhibit proliferation. It has been suggested that proliferation, which was observed at lower concentrations of phytoestrogens (<10 μM **[equivalent to ~2700 μg/L using molecular weight of genistein]**), was mediated through receptor responses, since proliferation was not stimulated by phytoestrogens in cells lacking ERs (reviewed in *(3)*).

2.2.9.1 Human

Estrogenic effects related to vaginal/endometrial/breast cytology and reproductive hormones have been examined in women, mostly post-menopausal, in a variety of studies after receiving soy diets or supplements (summarized in **Table 66**). Three of the four studies that assessed vaginal cytology did not report changes related to soy product intake *(289-291)*. The duration of soy product intake in these studies ranged from ~ 1 to 3 months. A fourth study that provided very limited detail and included alternating exposure to non-soy-based phytoestrogens reported increased vaginal cell maturation after women received soy flour supplements for 2 weeks *(292)*. A fifth study by Unfer *et al.*, 2004 with a longer exposure period (5 years) demonstrated estrogenic effects on the endometrium *(293)*. The Unfer *et al.*, 2004 study was cited by Sacks *et al.*, 2006 *(252)* as providing some cautionary evidence regarding the estrogenic activity of soy phytoestrogens because endometrial hyperplasia is considered a risk factor for cancer. However, a limitation of the Unfer *et al.*, 2004 *(293)* study noted in a letter to the editor by Foth and Nawroth *(294)* was that at baseline and at the 30-month evaluation period 25% of endometrium samples were inaccessible in the treated and placebo groups. **[The Expert Panel notes that 20–25% of endometrium samples were also inaccessible at the 5-year evaluation period.]** It does not appear that women with inaccessible endometrium samples at baseline were excluded for evaluation at future time points. Therefore, it is not known if endometrial hyperplasia was present at baseline in women with inaccessible endometrium samples. It was also noted that no information was provided about endometrial thickness or bleeding patterns. A sixth study reported increased proliferation of breast lobular epithelium and progesterone receptor expression in women who ate bread rolls containing 60 g soy supplement as textured vegetable protein *(295)*. The final report *(296)* with the full cohort of 84 individuals (including 33 added from a tissue bank) showed no differences between the control group and the group eating soy rolls other than plasma isoflavone levels.

Table 66. Summary of Studies Examining Estrogenicity Endpoints in Humans

Study Description[a]	*Endpoint*	*Effect*	*Reference*
For 93 days, postmenopausal women (n = 17–18) received soy protein powders providing total isoflavone intakes of 0.11 ± 0.01 (control), 1.00 ± 0.01 (low), or 2.00 ± 0.02 (high) mg/kg/day. **[Based on reported percentages of isoflavones, the diets resulted in intakes of about:** **• 0.06, 0.58, and 1.2 mg/kg/day genistein** **• 0.04, 0.33, and 0.66 mg/kg/day daidzein** **• 0.01, 0.09, and 0.18 mg/kg/day glycitein]**	• vaginal cytology • endometrial biopsy	No significant change	Duncan et al., 1999 *(289)*
	Plasma levels of: • 17β-estradiol • estrone • dehydroepiandrosterone-sulfate • sex hormone-binding globulin • insulin • thyroid-binding globulin	Slight decreases compared to baseline values, but the study authors stated effects were modest and unlikely to be of physiologic significance	
Postmenopausal women received a soy diet (n = 66) with soy products representing one-third of caloric intake or their usual diet (control group, n = 25) for 4 weeks. Foods were analyzed by HPLC, and it was determined that the soy diets provided 165 mg/day isoflavones **[40.3 mg/day genistein and 124.8 mg/day daidzein.]** **[Based on 58 kg bw: 0.69 mg/kg/day genistein and 2.2 mg/kg/day daidzein]**.	• vaginal cytology	No significant change	Baird et al., 1995 *(290)*
	Serum levels of: • FSH • LH • 17β-estradiol • sex hormone-binding globulin	No significant change	
Postmenopausal women were randomized to receive 46 g/day of either soy (n = 23) or wheat (n = 24) flour for 12 weeks.	• vaginal maturation index	No significant change	Murkies et al., 1995 *(291)*
In a study with Latin-square design, postmenopausal women (n = 23) were given 45 g soy flour, 10 g red clover sprouts, or 25 g linseed daily, during separate 2-week periods. Vaginal smears were taken after each supplementtion period, and cumulative effects of the 3 foods were compared at 6 weeks and at 2 and 8 weeks following supplementation.	• vaginal cytology	Significant changes in vaginal cytology after 6 weeks of supplementation; changes persisted for 2 weeks.	Wilcox et al., 1990 *(292)*
	• vaginal maturation index	Increased after soy supplementation	
	• FSH/LH levels	Concentrations remained higher than premenopausal values.	

Table 66 (continued)

Study Description[a]	*Endpoint*	*Effect*	*Reference*
Healthy menopausal women were randomized to receive for 5 years placebo (n=197) or 3 soy extract tablets/day (n=179), each containing 150 mg total isoflavones consisting of genistein 40–45%, daidzein 40–45%, and glycitein 10–20%.	• endometrial proliferation or hyperplasia	*Baseline:* Proliferative endometria in 2 women of the soy extract group and 3 women of the placebo group; hyperplasia was an exclusion criterion. *At 30 months:* No cases of proliferation or hyperplasia. *At 5 years:* No proliferation or hyperplasia in placebo group; 5 biopsies (3.2%) were proliferative and 6 biopsies (3.8%) were hyperplastic in the soy-extract group.	Unfer et al., 2004 *(293)*
Prior to scheduled biopsy, women with benign or malignant breast conditions but normal menstrual cycles were assigned to a control group (n=29) or a soy-supplement group (n=19). Women in the soy-supplement group ate bread rolls containing 60 g soy supplement as textured vegetable protein (45 mg isoflavones) for 14 days. Normal breast tissue was collected near lesions.	• proliferation of breast lobular epithelium • progesterone receptor expression	Both endpoints increased when controlled for stage of menstrual cycle and age in the preliminary study. The final report, with 33 tissue bank controls and some other subjects added, found no differences between the control group and the group eating soy rolls.	McMichael-Phillips 1998 *(295)* Hargreaves et al., 1999 *(296)*

FSH=follicle-stimulating hormone, LH=luteinizing hormone
[a] Isoflavone content assumed to be expressed in aglycone equivalents.

2.2.9.2 Experimental Animal Data

The rodent uterotrophic assay is the most commonly used *in vivo* bioassay for estrogenicity. Typically, this assay is conducted in immature or ovarectomized mature females to minimize potential interference from endogenous estrogens which may introduce variability in response and make the assay less sensitive if not controlled for. Other endpoints commonly used to assess estrogenicity in female experimental animals include uterine or vaginal epithelial cell height and cell proliferation, onset of puberty in females, and regularity of the estrous cycle.Only one study was identified that evaluated the uterotrophic response in sexually immature rats fed cow's milk or soy-based formulas. However, a relatively large number of studies have reported data on uterotrophic response in animals administered soy diets, mixtures of isoflavones, or genistein. A smaller number of studies have assessed daidzein and equol. No studies were identified that evaluated uterotrophic response following glycitein treatment.

Soy formula

Ashby *et al.*, 2000 *(297)* examined uterotropic effects of infant formulas. From PND 21/22 through PND 24/25, Alpk rats were given access to infant formula in drinking water bottles and RM1, a standard diet consisting of 6% soy protein. **[Effects on puberty were also examined and are discussed in Chapter 3b.]** Three soy-based and 1 cow-milk formulas were prepared as recommended by manufacturers. Rats fed the RM1 diet and not given infant formula served as negative controls, and rats given diethylstilbestrol or 17β-estradiol served as positive controls. Data were analyzed by analysis of covariance (ANCOVA). **[It is not clear how many animals were examined in each group, but if numbers above the bars in a graph indicated the numbers of animals, then each group contained 5–29 animals.]** Results are summarized in . Both cow-milk and soy-based formulas prepared at full strength increased uterine weight, with greater responses generally noted with recommended concentrations of soy compared to cow-milk formula (**Table 67**). Testing of 3 different concentrations of 1 of the soy formulas (Infasoy®) showed dose-related responses. It was noted that rats consumed Infasoy at a level 3 times the recommended intake for an infant on an mg/kg bw basis. The cow-milk formula, SMA Gold, and the 33%-strength Infasoy® formula were consumed at levels similar to those recommended for infants on a mg/kg bw basis. Faslodex, an ERα and ERβ antagonist, inhibited the uterotropic effects of soy and cow-milk formulas and of diethylstilbestrol **[data not shown for cow-milk formula]**. The aromatase inhibitor anastrozole attenuated the uterotropic response of Infasoy but not of 17β-estradiol **[data not shown]**. The gonadotropin-releasing hormone (GnRH) antagonist antarelix inhibited the uterotropic effect of cow-milk and soy formulas but not of diethylstilbestrol **[data not shown for cow-milk formula]**. Neither the Infasoy® nor cow-milk formula induced an uterotropic response in ovariectomized adult rats **[data not shown]**. The study authors concluded that both cow-milk and soy formulas induced estrogenic effects in rodents independent of formula phytoestrogen content. The authors further stated that infants fed recommended quantities of formulas would consume similar quantities of formula (on an mg/kg bw basis) as rats given the 33% soy formula or cow-milk formula, resulting in exposures at the threshold of estrogenic activity in rats.

Soy-based diet or soy extract

Results of estrogenicity testing in laboratory animals fed soy-based diets are summarized in **Table 68**. Reports of estrogenicity are mixed, with some studies in which rats or monkeys were fed soy-based diets reporting no effects on uterine size, morphomentric, or histological parameters [222; 298-300] and Thigpen *et al.*, 2007 reporting earlier onset of vaginal opening in $F_3$44 rats fed a high soy diet *(301)*. However, estrogenic effects occurred when soy isoflavones were added to a soy-free diet *(302-304)*. In the monkey studies *(222; 300)*, the genistein dose in the soybean extract was stated

Table 67. Uterotropic Responses in Rats Fed Infant Formulas or Diethylstilbestrol (Ashby et al., 2000)

Treatment	*Strength, % of recommended dilution for feeding infants*	*Uterine Wet Weight, % of negative control* [a]
Cow-milk formula		
SMA gold	100	132%[b]
Soy formula		
Wysoy	100	140%[c]
Farley's	100	125%[c]
Infasoy	33	116%
	100	148–179%[c]
	200	167%[c]
Diethylstilbestrol		
10 µg/L	N/A	156–196%[c]

N/A=Not applicable.
[a] Estimated from graphs by CERHR.
[b] $P<0.05$ compared to control.
[c] $P<0.01$ compared to control.
From Ashby et al., 2000 *(297)*.

to be equivalent to that of a woman receiving genistein 99.7 mg /day **[~ 2 mg/kg/day assuming a 50 kg bw]**.

A study in immature female mice found a soy-based diet to increase relative uterine weight and to attenuate the weight increase associated with feeding diethylstilbestrol, *(305)*. In a study in which rats were fed soy protein with and without the addition of an estrogen, there were no additive effects between soy diets and estrogen *(299)*. Soy diets that were not alcohol extracted to remove isoflavones inhibited the effects of estrogens on increased lactoferrin staining and epithelial luminal cell height. In some studies, increases in rodent uterine weight were greater in rats fed soy-free versus soy-containing diets *(297; 298; 306; 307)*.

As noted in Chapter 1, the amounts of individual isoflavones can vary considerably in soy extracts. These variations can lead to significant differences in estrogenic activity. A study by de Lima Toccafondo Vieira *et al.*, 2008 *(303)* compared uterotropic response in immature rats treated for 3 days with E_2 or one of five commercial samples of soy extract standardized to contain 40% total isoflavones. When the authors independently analyzed the isoflavone content of the samples they found that the actual percentage of total isoflavone ranged from 44 to 52% and that there was considerable variability in isoflavone composition. For example, the percentage as genistin and genistein varied from 0.8% to 3.13% and from non-detectable to 10.99%, respectively. Similarly the percentage as daidzin and daidzein ranged from 3.42% to 29.41% and 7.23% to 46.46%. The estrogenic potencies of the standardized soy extracts in the immature rat relative to E_2 ranged from 0.0009 to 0.005 (**Table 68**).

Variation in the phytoestrogen content of laboratory animal diets has been suggested to be a contributing factor to conflicting or inconsistent findings reported in experimental animal studies, especially for "low dose" studies of estrogenic compounds *(301; 308; 309)*. A general concern is that use of a

Table 68. Soy Estrogenicity in Laboratory Animals

Model	Design	Endpoint(s)	Results	Reference
Rat				
Alpk rat, PND 21(diet)	RM1 (standard) diet or AIN-76A (phytoestrogen-free diet) for 3 days. Some rats fed AIN-76A diet were also administered the anti-estrogen Faslodex.	• uterine weight	Higher in rats fed AIN-76A diet compared to RM1 diet, an effect that was eliminated with Faslodex treatment. The authors had no explanation for the results.	Ashby et al., 2000a *(298)*; abstract; Ashby et al., 2000b *(297)*
Wistar rats, PND 19 (gavage)	Gavage for 3 days beginning on PND 19 to one of the following groups: • vehicle control • dry soy extract doses of 125, 300,720, 1730, and 4150 mg/kg/day • E2 at 12.5, 30, 72, 173 and 415 mg/kg/day	• uterine weight	All extracts caused increases in uterine blotted weight with potencies relative to E2 of 0.0009 to 0.005	de Lima Toccafondo Vieira et al., 2008 *(303)*
$F_3$44 rat, 3 months old, OVX (gavage)	Gavage for 6 weeks (6 days a week) with: • vehicle, • soy extract (100 mg/kg/d) or • E2 (0.5 mg/kg/d)	• uterine weight • histopathology	No significant effects on uterine weight or histology in animals treated with soy extract (Increased uterine weight and hyperplasia observed in 17β-E2 group)	Gallo et al., 2008 *(310)*
Wistar rat, 3 months old, OVX (gavage)	Gavage for 21 days with soy extract doses (and isoflavone doses) of 10, 50, 100, 300, or 600 mg/kg/d soy extract. These dose levels corresponded to: • 4.3 mg/kg/d (21.3 mg/kg/d isoflavone) • 100 mg/kg/d (42.6 mg/kg/d isoflavone) • 300 mg/kg/d (127.8 mg/kg/d isoflavone) • 600 mg/kg/d (255.6 mg/kg/d isoflavone)	• uterine wet weight • endometrial morphometric measures • PCNA	Increased uterine weight and morphometric measures at soy extract of ≥ 100 mg/kg/d (42.6 mg/kg/d isoflavone)	Mosquette et al., 2007 *(304)*
$F_3$44 rat (diet)	Rats were fed a diet with isoflavone content (genistein + daidzein aglycone equivalents) considered: • low (< 7 µg/g diet for PMI 5K96) • medium (98 µg/g diet in batch 3 of PMI 5002) • high (223 or 431 µg/g diet in batches 1 and 2 of PMI 5002) beginning at weaning on PND 19	• day of vaginal opening	5.5-day earlier onset of vaginal opening in rats fed the diet containing the highest isoflavone content (32.6 versus 38.1 days)	Thigpen et al., 2007 *(301)*

Table 68 (continued)

Model	Design	Endpoint(s)	Results	Reference
F344 rat, 3 months old (diet)	For 14 weeks, rats (10–14/group) were fed either a: • casein-based diet, a diet containing 100 g/kg isolated soy protein (2.14 mg aglycones/g isoflavone) • diet containing 200 mg/kg isolated soy protein • casein-based diet containing 17.2 g/kg isoflavones (11.37 mg aglycones/g isoflavones) • casein-based diet containing 34.4 g/kg isoflavones	• uterine wet weight • histopathology	No significant effects	Nakai et al., 2005 *(311)*
Sprague Dawley rat, >40 days old, OVX (diet)	Soy diet containing 117.8 mg isoflavone/ 1800 calories or an alcohol-extracted soy diet with 11.6 mg isoflavone/1800 calories for 2 months.	• vaginal cytology • uterine weight • endometrial cell proliferation • lactoferrin expression • luminal epithelial cell height • apoptosis	No significant effects	Tansey et al., 1998 *(299)*
	Casein diets or soy diets containing low or high isoflavone levels (as described above) + conjugated equine estrogen at 0.313 or 0.625 mg/1800 calories.	• vaginal cytology • uterine weight • endothelial cell proliferation • apoptosis	Estrogen induced changes in all parameters examined. Soy isoflavones did not further affect these parameters.	
		• lactoferrin staining	High isoflavones attenuated staining induced by estrogen.	
		• luminal epithelial cell height	High isoflavones attenuated increase induced by estrogen	
CD Sprague-Dawley rat (diet)	Rats were fed a diet with isoflavone content (genistein + daidzein aglycone equivalents) considered: • low (< 7 µg/g diet for PMI 5K96), • medium (98 µg/g diet in batch 3 of PMI 5002), or • high (223 or 431 µg/g diet in batches 1 and 2 of PMI 5002) beginning at weaning on PND 19	• day of vaginal opening	No significant effects. Group averages for day of vaginal opening ranged from 31.9 to 32.7. **[No apparent dose-response for plasma concentrations of total isoflavones in rats fed PMI 5002 with medium or high isoflavone content.]**	Thigpen et al., 2007 *(301)*

Table 68 (continued)

Model	Design	Endpoint(s)	Results	Reference
Sprague-Dawley rat, (diet)	Multigeneration design using casein-based diet or alcohol-washed, isoflavone-poor soy protein diet. A commercial soy extract was added to the isoflavone-poor diet, providing isoflavone levels of 31.7–1046.6 mg/kg feed. Juvenile F_2 females were evaluated on PND 4. Some females were given SC ethinyl estradiol or bisphenol A from PND 21	• uterine weight • peroxidase • epithelial height	All 3 estrogenic endpoints were increased by the highest isoflavone diet (1046.6 mg/kg feed). There was no interaction with ethinyl estradiol or bisphenol A except additivity between ethinyl estradiol and isoflavones at the highest dietary level.	Wade et al., 2003 *(302)*
Mouse				
NMRI mouse, adult Exposed to DES as neonate and castrated in adulthood	Mice were fed soy-free diets or diets containing 7% roasted soy meal, for up to 10–20 days following castration. 17β-estradiol was given to some mice in each dietary group.	• prostatic metaplastic transformation • expression of c-*fos* oncogene *(endpoints induced by 17β-estradiol in the same study)*	Soy diets did not affect either endpoint and did not alter estrogenic effects in mice exposed to 17β-estradiol.	Mäkelä et al., 1995 *(312)*
Han-NMRI mouse, PND 16	Mice weaned at 16 days old to soy-free diet or diet containing 7% roasted soy meal. Diethylstilbestrol was added to some diets (6 µg/kg **[kg feed assumed]**).	• relative uterine weight after 7 days on diet	Relative uterine weight increased 10–15% by soy diet. Soy diet decreased the diethylstilbestrol-associated increase in relative uterine weight.	Mäkelä et al., 1995 *(305)*
CD-1 mouse, PND 15	Mice were weaned at 15 days of age and fed 1 of the following diets for 3, 5, or 7 days: • Rodent Chow #5002 (no information on dietary components) • Rodent Chow 5001 (reported to have high isoflavone levels and assumed to be soy based) • Mouse Chow #5015 (reported to have high isoflavone levels and assumed to be soy based) • NIH-07 (12% soybean meal) • NIH-31 (5% soybean meal) • AIN-76A (casein based)	• uterine:body weight ratios	Compared to the 5002 diet, uterine weight:body weight ratios were higher with the 5015 diet, NIH 31 diet, and the AIN-76A diet on days 3, 5, and 7. No significant increases in uterine weight were noted for the 5001 or the NIH-07 diets compared to the 5002 diet.	Thigpen et al., 1987 *(306)* Thigpen et al., 1999 *(307)*

Table 68 (continued)

Model	Design	Endpoint(s)	Results	Reference
Other Species				
Adult cynomolgus (*Macaca fascicularis*) monkey, OVX	Monkeys (n=12) were fed a soy protein isolate diet providing a dose of 26.6 mg free genistein/monkey/day (the equivalent of a women receiving 99.7 mg genistein/day **[~2 mg/kg/day assuming a 58 kg bw]**[a]). A control group (n=13) was given isoflavone-extracted soy diet and a positive control group the extracted soy diet supplemented with estrogen (n=15). Animals were fed the diets for 6 months.	• vaginal maturation • karyopyknotic indices	Not significantly affected by soy diet	Cline et al., 1996 *(222)*
	For 36 months, monkeys (n=57–62/group) were fed: • soy protein isolate that was alcohol treated to remove isoflavones (negative control) • untreated soy protein isolate (~91 mg genistein, 31 mg daidzein, and 7 mg glycitein) • alcohol-extracted soy protein isolate containing conjugated equine estrogens (positive control)	• breast and uterine proliferation • sex steroid receptor expression • serum estrogen level	In the soy protein isolate group, there was no increase in breast or uterine proliferation or steroid receptor expression. Mammary gland thickness and serum estrone and 17β-estradiol levels were significantly reduced.	Wood et al., 2004 *(300)*

DES=Diethylstilbestrol.

[a]Assumptions used in dose estimates obtained from *(313)*.

diet with high phytoestrogen content may decrease the sensitivity of the animal model to detect potential estrogenic activity due to the exposure under study. Jensen *et al.*, 2007 summarized the isoflavone content of a wide number of commonly used rodent diets. Diets formulated without soy had a low isoflavone content, <50 µg/g chow and diets formulated with soy had much higher isoflavone contents, typically ranging between ~ 75–500 µg/g chow. Most soy meal-based commercial diets for rodents result in a typical isoflavone exposure of 80 to 160 mg/kg/day, a range that is much higher than estimated human exposures (reviewed in *(301)*).

As a further complication, phytoestrogen content of the same rodent diet can vary from batch-to-batch *(301; 309)*, potentially altering experimental outcomes and contributing to variability in response observed within a laboratory or between laboratories. For example, Thigpen *et al.*, 2007 assessed age at vaginal opening in $F_3$44 and CD Sprague-Dawley rats fed one of three batches, i.e., different mill dates, of PMI 5002 *(301)*. The isoflavone content of genistein + daidzein in the three batches were 98, 223, and 431 µg/g diet and the corresponding average day of vaginal opening in $F_3$44 rats were 35.5, 33.9, and 32.6 days. The earlier onset of vaginal opening in the diet containing 431 µg/g diet was statistically significant. By way of comparison, the mean day of vaginal opening occurred on 38.1 days in $F_3$44 rats fed PMI 5K96, a casein-based diet containing only trace levels of phytoestrogen. In contrast, the average day of vaginal opening did not differ significantly in the Sprague-Dawley rats fed either the PMI 5K96 or any batch of PMI 5002 (the means for these four groups ranged from 31.9 to 32.7 days). **[Figure 3 in this paper shows that plasma concentrations of total isoflavones in SD rats were much greater in animals fed the PMI 5002 diet compared to PMI 5K96, but that isoflavone concentration in animals fed PMI 5002 did not appear to track with the relative isoflavone ranking of the diets.]** Because differences in vaginal opening based on the phytoestrogen content of the diet were observed more frequently for CD-1 mice and $F_3$44 rats compared to CD Sprague-Dawley rats, Thigpen *et al.*, 2007 proposed that the CD Sprague-Dawley rat is not the ideal model to evaluate estrogenic activities (**Table 68**). In addition, Thigpen and others have suggested that an open-formula diet low in phytoestrogens, i.e., containing no more than 20 µg/g diet of total genistein equivalents, be used to enhance the sensitivity of estrogenic bioassays *(301; 308)* **[Ingredients in open-formula diets are quantified and publically accessible. In closed-formula diets the ingredients are listed but the specific concentrations are unknown or considered proprietary.]** Ideally, the diet should also be low in metabolizable energy (~ 3.1 Kcal/g diet) because high levels of metabolizable energy can alter estrogenic response independent of phytoestrogens.

Genistein

A relatively large number of studies have assessed the *in vivo* estrogenicity of genistein (**Table 69**). Oral exposure studies in rats were inconsistent, with one study demonstrating an increase in uterine weight following oral exposure of rats to ≥150 ppm **[~14 mg/kg/day]** genistein through diet, but other studies indicating no effect on uterine weight with genistein doses up to 750 ppm in feed **[~124 mg/kg/day]**. Thigpen *et al.*, 2007 reported earlier onset of vaginal opening in $F_3$44 fed genistein in the diet at 300 or 400 µg/g feed **[~30 to 65 mg/kg/day starting]** at weaning *(301)*. Uterine weight was increased in most studies in which rats were exposed to ≥2 mg/kg/day genistein by sc or ip injection. In oral dosing studies of mice, increases in uterine weight were observed following exposure to **[≥200 mg/kg/day]** genistein through diet or by gavage. Uterine weights of mice were consistently increased following sc dosing with ≥5 mg/kg/day genistein. Potency of genistein in inducing increases in uterine weight was much lower than that of 17β-estradiol or diethylstilbestrol.

Table 69. Genistein Estrogenicity in Laboratory Animals

Animal model	*Design*	*Endpoint*	*Results*	*Reference*
Rat – Oral				
Sprague Dawley rat, lactating, OVX (diet)	Dietary genistein added to feed at 0, 0.5, 1.6, or 5 mg/day for 2 weeks. **[Phytoestrogen content of feed was not reported. Based on reported body weights (~325 g), genistein intake was estimated at 0, 1.5, 4.9, and 15 mg/kg/day.]**	• uterine dry weight	No effect of genistein treatment on the mature rat uterus.	Anderson et al., 1998 *(315)*
Sprague Dawley rat, 21-days-old (diet)	Mothers of rats were fed AIN-76A (a phyto-estrogen-free diet), AIN-76A + genistein 250 mg/kg feed, or 17β-estradiol 250 µg/kg feed from conception through PND 21. **[A 350 g female eating 28 mg feed/day would consume 20 mg/kg/day genistein.]**	• uterine weight • ERα expression	No significant differences	Cotroneo et al., 2001 *(215)*
Sprague Dawley rat, 60 days old, OVX (diet)	Dietary genistein added to modified AIN-76 feed × 5 days at 0, 150, 375, or 750 ppm **[~14, 35, 71 mg/kg/day based on actual body weights and estimated feed intake**a**]**. Compared to diets containing 17β-estradiol 0.5, 1.0, and 1.5 ppm.	• uterine weight	*Wet Weight:* Increased by genistein 375 and 750 ppm. Potency about 0.13% that of 17β-estradiol *Dry Weight:* Increased at all genistein exposure levels. Potency about 0.13% that of 17β-estradiol.	Santell et al., 1997 *(211)*
	Dietary 17β-estradiol 1 ppm was given alone or with genistein at the above levels, × 21 days.	• uterine wet weight • plasma prolactin • mammary growth	There was no antagonism of 17β-estradiol by genistein co-treatment.	
	Dietary genistein 750 ppm **[~71 mg/kg/day]**, 17β-estradiol 1 ppm, or untreated AIN-76 feed × 21 days.	• northern blot of *c-fos* from homogenized uteri	Both genistein and 17β-estradiol increased *c-fos* RNA.	
Sprague Dawley rat, 30 days old, OVX (diet)	Dietary genistein added to AIN-93G feed at 0, 375, or 750 ppm **[~62 and 124 mg/kg/day based on actual body weights and estimated feed intakes**a**]** × 13 days	• uterine wet weight	No effect of genistein treatment on the immature uterus.	
Wistar rat, juvenile, OVX (gavage)	100 mg/kg of genistein or 30 or 100 µg/kg ethinyl estradiol (EE) for 3 days	• uterine wet weight • uterine and vaginal epi-thelial height	Increase in all measures by genistein and ethinyl estradiol treatments (potency ~75–80% of 30 µg/kg EE)	Schmidt et al., 2006 *(316)*

Table 69 (continued)

Animal model	Design	Endpoint	Results	Reference
Female $F_3$44 rat (diet)	Dietary genistein added to AIN-76 feed at 150, 300, or 400 µg/g feed **[~14 to 20, 30 to 44, and 45 to 65 mg/kg/day based on actual body weights and feed consumption]** beginning at weaning on PND 19.	• day of vaginal opening	Significantly earlier onset of vaginal opening in rats fed AIN-76A supplemented with 2 highest doses of genistein (34 and 26.8 days) compared to control AIN-76A (36.8 days).	Thigpen et al., 2007 *(301)*
CD Sprague Dawley rat (diet)			Significantly earlier onset of vaginal opening in rats fed AIN-76A supplemented with highest dose of genistein (27.1 days) compared to control AIN-76A (29.6 days)	
Rat–Injection				
Sprague Dawley rat, 23 days old (SC injection)	Rats SC injected with 500 mg/kg bw genistein. 500 µg/kg bw estradiol benzoate was positive control.	• uterine wet and dry weight • epithelial cell height • PCNA staining • expression of estrogen, progesterone, and EGF receptors	Increased at a dose 1000 times higher than the estradiol benzoate dose producing the same effects. Changes in expression were similar for genistein and estradiol benzoate (e.g., ↓estrogen receptor, ↑progesterone, and EGF but not phosphorylated EGF receptor expression), suggesting a similar mechanism of action.	Cotroneo et al., 2005 *(318)*
Wistar rat, juvenile, intact and OVX (SC injection)	10 mg/kg/day genistein and 1 or 4 µg/kg bw/day of 17β-estradiol for 3 days	• uterine wet weight • epithelial height • PCNA cells (% positive)	Significant ~1.3-fold increase in uterine weight compared to OVX control (~5 to 5.5-fold increase for 1 or 4 µg/kg/d E2). No effect on epithelial height compared to OVX control. Decrease protein and mRNA PCNA expression in OVX animals. (No effect in intact animals compared to control)	Diel et al., 2006 *(319)*
Crj:CD (DD) rat, 20 days old (SC injection)	Rats SC injected with 1, 5, or 20 mg/kg/day genistein daily for 3 days on regular or low-phytoestrogen diet.	Uterine weight: • absolute and relative • wet and blotted	Increased at 20 mg/kg/day. Not affected by diet.	Yamasaki et al., 2002 *(326)*
C57BL/6 mice, 10-weeks old, OVX (SC injection)	25 mg/kg/day of genistein or 5µg/kg/d of 17β-E2 on 5 days a week for 4 weeks	• uterine wet weight • histology	Increased uterine weight compared to OVX animals (17β-E2 > genistein). Degenerative changes and apoptosis in endometrium of genistein-treated animals (disordered proliferative endometrium in 17β-E2 animals)	Garcia-Perez et al., 2006 *(320)*

Table 69 (continued)

Animal model	*Design*	*Endpoint*	*Results*	*Reference*
Long Evans rat (SC injection)	10 mg/kg/day genistein or 50 µg estradiol benzoate from PND 0 to PND 3 (4 treatments); dams fed phytoestrogen-free diet of AIN-93G	• day of vaginal opening	Significant earlier onset of vaginal opening, ~1.5 days	Bateman and Patisaul, 2009 *(317)*
Sprague Dawley rat, 20 days old (SC injection)	Rats SC injected with genistein 35 mg/kg/day for 3 days.	• uterine wet and blotted weight • vaginal weight	Increased to 64–71% of the weight achieved with ethinyl estradiol 0.3 µg/kg/day. Increased to 64% of the weight achieved with ethinyl estradiol 0.3 µg/kg/day.	Kim et al., 2005 *(321)*
Wistar rat, OVX (SC injection)	Rats were fed soy-free diets and SC injected with genistein 0.0025, 0.025, 0.25, or 2.5 mg/kg/day for 7 days.	• uterine weight • estrogen-related morphologic changes in uterus	No dose-related effects. However, the uterine epithelium was slightly taller and retained columnar structures compared to atrophic changes in control animals.	Mäkelä et al., 1999 *(322)*
Sprague Dawley rat, 16 days old (SC injection)	Rats SC injected with 500 mg/kg bw genistein on PND 16, 18, and 20.	• uterine weight	Increased at 22 days of age but not at 33 or 50 days of age. **[This finding demonstrates that the effect is highly reversible.]**	Murrill et al., 1996 *(323)*
Holtzman rat, OVX (IP injection)	Rats injected IP with 400 µg genistein **[2 mg/kg bw based on actual body weight]**.	• uterine wet weight • protein and phospholipid synthesis	Increased at 6 hours following treatment.	Noteboom and Gorski, 1963 *(324)*
Wistar rat, 3 months old, OVX (SC injection)	Rats SC injected with 0.31, 0.62, 1.25, 2.50, or 5.00 mg **[1.5, 3, 6, 12, or 24 mg/kg bw]** and evaluated 6 hours later.	• uterine weight	Increased at ≥0.62 mg/rat **[3 mg/kg bw]**.	Perel and Lindner, 1970 *(325)*
	Rats SC injected with 0.62, 1.25, or 2.50 mg/day **[3, 6, or 12 mg/kg/day]** for 3.5 days.	• uterine weight	Increased at ≥1.25 mg/rat/day **[6 mg/kg/day]**.	
Mouse-Oral				
Mouse, 19–21 days old (diet)	Mice fed genistein in the diet for 4–6 days. Total genistein doses received were 5–20 mg **[~100–400 mg/kg/day based on assumed body weight of 0.01 kg]**[a].	• uterine wet weight	Increased by 8 mg genistein. Potency about 0.001% that of diethylstilbestrol.	Bickoff et al.,1962 *(327)*
Mouse, immature (diet)	Genistein administered through diet at 2.5 and 5.0 mg/day for 4 days **[250 and 500 mg/kg/day, assuming that the mice weighed ~10 g as in a previous study with hay extract]**.	• Uterine weight	Increased at 2.5 mg/day. Genistin at ≥2.5 mg/kg bw in diet also increased uterine weights but was less potent than genistein.	Cheng et al., 1955 *(328)*

Table 69 (continued)

Animal model	*Design*	*Endpoint*	*Results*	*Reference*
Swiss CD-1 mouse 21–22 days old (gavage)	Mice were gavaged with genistein 4 times/day for 4 days for a total dose of 6 or 8 mg/mouse **[~150 or 200 mg/kg/day]**.	• uterine wet weight, uncorrected or corrected for initial body weight	No treatment effect.	Farmakalidis and Murphy, 1984 *(329)*
B6D2F$_1$ mouse **[age not specified, but apparently weanlings based on body weight]** (gavage)	Mice were gavaged with a total 8 mg genistein administered in 4 daily doses **[200 mg/kg/day based on actual body weight]**.	• uterine weight	Increased, potency 0.001% that of diethylstilbestrol. Genistin administered at equimolar concentration (12 mg) also increased uterine weight with a potency similar to that reported for genistein.	Farmakalidis et al., 1985 *(330)*
Mouse, immature (diet)	Injected for 3 days with doses ranging from 0.0001 to 1000 mg/kg/day.	• relative uterine wet weight • uterine epithelial cell height • gland number • lactoferrin intensity	Uterine weight increased at >10 mg/kg/day, with potency 0.1% that of 17β-estradiol. Cell height increased at >10 mg/kg/day, with potency 0.02% that of 17β-estradiol. Gland number increased with maximum response observed at 50 mg/kg/day; potency 0.2% that of 17β-estradiol. Lactoferrin intensity increased at >10 mg/kg/day.	Jefferson et al., 2002 *(331)*
CD-1 mice (diet)	Dietary genistein added to AIN-76 feed at 150, 300, or 400 µg/g feed beginning at weaning on PND 19	• day of vaginal opening	Significant 2.5 to 5.6 day earlier onset of vaginal opening in mice fed AIN-76A supplemented with any dose of genistein (22.9, 21, and 19.8 days) compared to control AIN-76A (25.4 days).	Thigpen et al., 2007 *(301)*
ddy Mouse, 8 weeks old, OVX (diet)	Mice fed an AIN-93G diet and SC injected with 0.7 mg/day genistein for 4 weeks **[22 mg/kg/day based on actual body weight]**.	• histological evaluation of uterus	Phenotypes of epithelial cells were not affected at 0.7 mg/day.	Ishimi et al., 2000 *(332)*
	Mice fed an AIN-93G diet and SC injected with 0.7, 2, or 5 mg/day genistein for 2 weeks **[22, 63 or 156 mg/kg/day]**.	• uterine weight	Slight increase at 2 mg/day and marked increase at 5 mg/kg day.	
ddy Mouse, 8 weeks old, intact (diet)	Mice fed an AIN-93G diet and SC injected 2 or 5 mg/day genistein for 2 weeks **[63 or 156 mg/kg/day]**.	• uterine weight	Increased at 5 mg/day.	

Table 69 (continued)

Animal model	*Design*	*Endpoint*	*Results*	*Reference*
$B6D2F_1$ mouse, 22 days old (gavage)	Mice were gavaged with genistein 4 times/day for 4 days. Total genistein dose 12 mg/mouse **[~300 mg/kg/day]**.	• uterine wet weight • ER binding	Uterine weight increased with 0.001% that of diethylstilbestrol. Receptor binding 2–3 orders of magnitude lower than that of 17β-estradiol **[estimated from graph].**	Song et al., 1999 *(333)*
Mouse-Injection				
BSVS mouse, 3–4 weeks old (SC injection)	Mice injected SC twice daily for 3 days with estrone, genistein, or both. Total doses 800 and 1600 µg genistein + 0.025, 0.1, and 0.4 µg estrone. **[Genistein doses estimated at 27 and 53 mg/kg/day based on assumed weanling body weight of 0.01 kg.]**a	• uterine and vaginal wet weight	Increased by estrone or genistein administered alone. At 0.025 µg estrone, genistein did not change or slightly increased estrone response; at ≥0.1 µg estrone, genistein attenuated estrone response.[b] **[Responses are additive at low doses and antagonistic at high doses of estrogens.]**b	Folman and Pope, 1966 *(314)*
	Mice injected SC twice daily for 3 days with estriol or genistein or mixture of the 2 compounds. Total doses were 1600 and 5000 µg genistein + 2 or 40 µg estriol. **[Genistein doses estimated at 53 and 167 mg/kg/day.]**	• uterine and vaginal wet weight	Increased by estriol or genistein administered alone. Estriol responses at 2 µg were augmented by 5000 µg genistein.b	
	Mice injected SC twice daily for 3 days with diethylstilbestrol or genistein or mixture of the 2 compounds. Total doses were 1600 and 5000 µg genistein + 0.02 or 0.08 µg diethylstilbestrol. **[Genistein doses estimated at 53 and 167 mg/kg/day.]**	• uterine wet weight • vaginal wet weight	Increased by diethylstilbestrol or genistein administered alone. At 0.08 µg diethylstilbestrol, 1600 µg genistein attenuated diethylstilbestrol response. At 0.02 µg diethylstilbestrol, 5000 µg genistein augmented diethylstilbestrol response.[b] **[Responses are additive at low doses and antagonistic at high doses of estrogens.]**b	

Table 69 (continued)

Animal model	*Design*	*Endpoint*	*Results*	*Reference*
Mouse, immature (SC injection)	Mice were SC injected with genistein 1 or 2 mg/day for 4 days **[100 or 200 mg/kg/day]**.	• uterine weight	Increased at 1 mg/day with potency of 0.002% that of diethylstilbestrol. Genistin at ≥2.5 mg/kg bw in diet and 2 mg by SC injection also increased uterine weights but was less potent than genistein[b]	Cheng et al., 1955 *(328)*
CD-1 mouse, 5 days old (SC injection)	Injected on PND 1–5 with 50 mg/kg/day.	• relative uterine weight	Increased with potency ~0.002% that of diethylstilbestrol.	Newbold et al., 2001 *(334)*

RNA=ribonucleic acid; EGF=epidermal growth factor; PCNA=proliferating cell nuclear antigen, OVX=ovariectomized; E2=estradiol.
[a] Assumptions used in dose estimates obtained from EPA *(313)*.
[b] Statistical analysis not clearly indicated; only obvious effects are listed.

Other estrogenicity endpoints observed with genistein exposure included increased epithelial cell height and uterine gland numbers.

One study *(314)* reported that genistein (≥800 µg **[~27 mg/kg/day]**) administered by sc injection to mice could either attenuate or augment the estrogenic responses of potent estrogens, depending on the doses of both compounds. In contrast, a second study *(211)* demonstrated that genistein did not inhibit 17β-estradiol responses when fed to rats at concentrations up to 750 ppm **[~71 mg/kg/day]** in diet. **[This finding is most likely due to the fact that the free fraction of the aglycone is much lower after oral than after sc administration in spite of the higher oral dose.]**

A number of studies have compared uterotrophic response in rodents treated with genistein or genistin, which is the sugar conjugated form of genistein and the predominant form of the isoflavone found in soy formula. Most recently, Jefferson *et al.*, 2009 *(217)* treated female CD-1 mice with oral doses of 0, 6.25, 12.5, 25, 37.5 mg/kg bw/day of genistin or 0, 25, 37.5, 75 mg/kg bw/day genistein. The oral doses of genistin are presented as agylcone equivalents and the uncorrected administered doses of genistin were 0, 10, 20, 40, or 60 mg/kg bw/day. In this study, genistin elicited a more potent uterotrophic response than genistein, causing significant increases in uterine weight at ≥25 mg/kg bw/day compared to 75 mg/kg bw/day for genistein. In addition, the authors considered the genistin response similar to that observed following neonatal sc injection with 12.5, 20, or 25 mg/kg bw/day genistein where a significant uterotrophic response at ≥20 mg/kg bw/day. Other studies that have characterized uterotrophic response to genistein report increases in uterine weight with potencies less than or equal to those of genistein (**Table 69**).

Daidzein

In contrast to genistein, a smaller number of studies have assessed the classic *in vivo* indicators of estrogenicity following treatment with daidzein. Those studies are discussed below and summarized in **Table 71** and often include a genistein treatment group in addition to a positive control group. With the exception of one study reporting a small, but statistically significant, uterotrophic response after oral treatment with daidzin, daidzein was not reported as uterotrophic in other studies following treatment by oral or injection routes of administration.

Jefferson *et al.*, 2002 *(331)* compared the estrogenic activity of daidzein, genistein, DES, and 17β-estradiol using a transcriptional activation assay of ER using human ovarian carcinoma cells that were stably transfected with an estrogen responsive luciferase reporter gene plasmid (BG1Luc4E2) and *in vivo* indicators of estrogenic response, i.e., uterine stimulation, epithelial cell height, gland number and lactoferrin protein. The *in vivo* measures were assessed in female CD-1 mice treated for three days starting on PND 17 with sc injections of 0.01 to 1,000,000 µg/kg/day for each compound. DES and 17β-estradiol were uterotrophic and caused significant increases in uterine epithelial cell height at doses of ≥1 µg/kg/day and genistein at doses of ≥100,000 µg/kg/day. Daidzein did not increase in either uterine weight or epithelial cell height at any dose level and was overall less estrogenic than genistein (**Table 70**).

Heikaus *et al.*, 2002 *(335)* compared the effects of sc injection of genistein and daidzein on a number of endometrial genes as indicators of estrogenic effects. They concluded that genistein displays a higher short-term estrogenic potency *in vivo* compared to daidzein. In this study, a single sc injection

Table 70. Comparison of Estrogenicity for Genistein, Daidzein, Diethylstilbestrol, and 17β-Estradiol (Jefferson et al., 2002)

Chemical	*Reporter Assay, EC50 (µg/ml)*	*Uterotrophic Assay*	*Uterine Epithelial Cell Height*	*Uterine Gland Number*	*Lactoferrin*
17β-Estradiol	0.0000023	++++	++++	++++	++++
Diethylstilbestrol	0.0000049	++++	++++	++++	++++
Genistein	0.19	+++	++	+++	+++
Daidzein	0.52	–	–	+++	–

From Jefferson et al., 2002 *(331)*.

of 2.5 mg genistein to adult ovariectomized Sprague Dawley rats resulted in increased expression of a gap junction connexin (Cx) gene, Cx26, within 24 hours, an effect that could be suppressed by co-administration of ICI 182.780. Treatment with the same amount of daidzein did not affect Cx26 gene expression and neither genistein nor daidzein affected any of the other endometrial genes tested (Cx43, clusterin, and complement C3).

Akbas *et al.*, 2007 *(336)* compared the uterotrophic response to estradiol (0.5 mg/kg), genistein (2 mg/kg), and daidzein (2 mg/kg) in ovariectomized mice 8-hours following treatment by ip injection. In this study, only estradiol and genistein induced a significant increase in uterine weight compared to the ovariectomized control group. The authors also reported that adult treatment with genistein, but not daidzein, regulated uterine HOXA10 mRNA. In this study, genistein treatment induced a ~1.5-fold increase in HOXA10 mRNA expression whereas E2 repressed expression to a level that was ~50% of control. Neither isoflavone had a lasting effect on HOXA10 expression when administered *in utero*. Also, reporter gene expression regulated by HOXA10 estrogen response element (ERE) was increased by genistein but not daidzein in Ishikawa cells transfected with the pGL3 promotor/HOXA10 ERE construct.

Farmakalidis *(330)* compared the uterotropic activity in $B6D2F_1$ mice orally treated with 0.0012 mg DES, 8 mg genistein, 12 mg genistin, or 12 mg daidzin **[These dose levels are referred to as total doses of the compounds administered (in four daily doses) per mouse.]**. When scaled to a dose level of 1 mg, the uterotropic activity of 1.5 mg genistin was equal to that of 1 mg genistein, giving a 1:1 molar relationship in oestrogenic activity between genistin and genistein oral. In a previous study using CD-1 mice, 4-days of gavage treatment with 6 mg genistein, 8 mg genistein, or 12 mg genistin did not cause significant increases in uterine weight *(337)*. These doses of genistein were reported to be estrogenic by Bickoff *et al.*, 1962 (as cited in Farmakalidis *et al.*, 1984 *(337)*). However, treatment with 12 mg daidzin caused a statistically significant 15% increase in body weight corrected uterine weight compared to control weight (28.9 mg versus 25.1 mg). A uterotrophic response was also observed in the positive control groups where animals were treated with 0.6 µg or 0.8 µg DES. The authors noted that these doses of DES were ~ 10 times higher than doses used by another researcher, Bickoff *et al.*, 1962 (as cited in Farmakalidis *et al.*, 1984) as they were unable to elicit a uterotrophic response at the lower dose level used by Bickoff *et al.*, 1962. The response to DES and the lack of uterotrophic response to genistein led the authors to conclude that the CD-1 mouse was less responsive to estrogens compared to other strains.

Table 71. Uterotrophic Response in Laboratory Animals Treated with Genistein, Daidzein, or Equol

Animal Model	Isoflavone	Design	n	Endpoint	Results	Reference
Genistein and Daidzein						
CD-1 mice Adult, OVX	Genistein (IP injection)	2 mg/kg	3	Uterotrophic response at 8 hours	~1.4-fold increase compared to OVX controls	Akbas et al., 2007 *(336)*
	Daidzein (IP injection)	2 mg/kg	3		No effect	
	Estradiol (IP injection)	0.5 mg/kg	3		~1.5-fold increase compared to OVX controls	
CD-1 mice PND 20/21–23/24	Genistin (gavage)	12 mg total (over 4 daily doses)	8	Uterotrophic response (PND 24/25)	No effect	Farmakalidis and Whitten, 1984 *(337)*
		6 or 8 mg total (over 4 daily doses)	13–16		No effect	
	Daidzin (gavage)	12 mg total (over 4 daily doses)	8		1.2-fold increase compared to control (based on uterine weight corrected for body weight at weaning)	
	DES (gavage)	0.0006 or 0.0008 mg total (over 4 daily doses)	11–15		3.1-fold increase	
CD-1 mice PND 17–19	Genistein (SC injection)	0.01 to 1,000,000 µg/kg/day	≥5	Uterotrophic response (PND 20)	≥100,000 µg/kg/day Magnitude of effect at 500,000 µg/kg was similar to DES response at 10 µg/kg and 17β-^{E}2 at 500 µg/kg	Jefferson et al., 2002 *(331)*
	Daidzein (SC injection)		≥5		No effect	
	DES (SC injection)		≥5		≥1 µg/kg/day	
	17β-Estradiol (SC injection)		≥5		≥1 µg/kg/day	

Table 71 (continued)

Animal Model	Isoflavone	Design	n	Endpoint	Results	Reference
B6D2F$_1$ mice PND 20/21–23/24	Genistin (gavage)	12 mg total (over 4 daily doses)	26	Uterotrophic response (PND 24/25)	1.38-fold increase compared to control (Relative potency to genistein = 0.66)	Farmakalidis et al., 1985 *(330)*
		8 mg total (over 4 daily doses)	7		1.42-fold increase compared to control (Relative potency arbitrary assigned as 1.00)	
	Daidzin (gavage)	12 mg total (over 4 daily doses)	16		No effect (Relative potency to genistein = 0.26)	
	DES (gavage)	0.0012 mg total (over 4 daily doses)	24		1.79-fold increase compared to control (Relative potency to genistein = 100,000)	
Wistar rats Adult, OVX	Genistein (diet)	42 mg/kg/day and < 0.1 mg/kg/day daidzein for 12 weeks	7	Uterotrophic response	No effect	Hertrampf et al., 2009 *(343)*
	Genistein (SC injection)	10 mg/kg/day for 12 weeks	7		~2.5-fold increase	
	Genistein and daidzein enriched diet	14 mg/kg/day genistein and daidzein for 12 weeks	7		No effect	
	17β-estradiol	2 µg/kg/day for 12 weeks	7		~5.0-fold increase	
Genistein and Equol						
Long Evans rat PND 0–3	Genistein (SC injection)	10 mg/kg	7–8	• Vaginal opening • Estrous cycling	• ~1.3 day acceleration in vaginal opening • 71% with irregular estrous cycles by 10 weeks (either persistent estrous or diestrus)	Bateman and Patisaul, 2009 *(317)*
	Equol (SC injection)		8–10		• No effect on age at vaginal opening • 75% with irregular estrous cycles by 10 weeks (either persistent estrous or diestrus)	
	Estradiol benzoate (SC injection)	50 µg	10–15		• ~5 day acceleration in vaginal opening • 100% in persistent estrous by 10 weeks	

Table 71 (continued)

Animal Model	*Isoflavone*	*Design*	*n*	*Endpoint*	*Results*	*Reference*
$B6D2F_1$ mice PND 17–20	Genistein (gavage)	100 mg/kg	4–5	Uterotrophic response (PND 21)	~3-fold increase compared to control	Brienholt et al., 2000 *(342)*
	Equol (gavage)	100 mg/kg	4–5		~2-fold increase compared to control	
	17β-Estradiol (gavage)	5 mg/kg	4–5		~4 -fold increase compared to control	
Equol						
Sprague Dawley rats	Equol (SC injection)	1, 10, 100, or 1000 µg/pup from PND 1–5	≥7	Uterotrophic response (PND 5)	1000 µg/pup for dry weight (No effect based on wet weight)	Medlock et al., 1995 *(338)*
	DES (SC injection)	0.001, 0.01, 0.1, 10, or 100 µg/pup from PND 1–5	≥7		≥0.01 µg/pup for wet or dry weight	
	Equol (SC injection)	10, 100, or 1000 µg/pup from PND 10–14	≥7	At PND 14: • Uterotrophic response • Uterine gland number • Luminal epithelial height	*10 µg/pup:* • No effects *100 and 1000 µg/pup:* • No effects on uterine weight • No effects on luminal epithelial height • ↓ in gland number	
	DES (SC injection)	10 µg/pup from PND 10–14	≥7		*10 µg/pup:* • ~3.6-fold increase in uterine weight • ↓ uterine gland number • ↑ luminal epithelial height	
NMRI mice PND 15–21	Equol (drinking water)	50 µg/ml	10	Uterotrophic response (PND 22)	No effect	Nielsen et al., 2009 *(339)*
	"Low" or "high" equol milk (oral)	"Low" or "high" equol milk	15		No effect	
	17β-Estradiol (drinking water)	33 or 100 ng/ml	8		33 ng/ml: ~3-fold increase 100 ng/ml: ~5-fold increase	

Table 71 (continued)

Animal Model	*Isoflavone*	*Design*	*n*	*Endpoint*	*Results*	*Reference*
Sprague Dawley OVX rats	Equol (diet)	400 mg/kg for 6–7 weeks	10	Uterotrophic response	1.3-fold increase *Authors note that previous experiments with lower dose levels did not show estrogenic effects on the uterus*	Rachon et al., 2007 *(340)*
	Estradiol-benzoate (diet)	10 mg/kg for 6–7 weeks	10		4.5-fold increase	
C57BL/6 OVX mice	Equol (SC injection)	4, 8, 12, or 20 mg/kg/day to PND 30 for 12 days	5	• Uterotrophic response • Uterine epithelial proliferation • Vaginal epithelial height	*4 mg/kg/day:* No effects *8 mg/kg/day:* • No statistically significant effect on uterine weight • ↑ Uterine epithelial proliferation • ↑ Vaginal epithelial height *12 mg/kg/day:* • 2-fold increase in uterine weight • ↑ Uterine epithelial proliferation • ↑ Vaginal epithelial height *20 mg/kg/day:* • 3.5-fold increase in uterine weight • ↑ Uterine epithelial proliferation • ↑ Vaginal epithelial height	Selvaraj et al., 2004 *(341)*
	Equol (diet)	500 or 1000 ppm to PND 30 for 12 days	5		*500 ppm:* • No effect on uterine weight • ↑ Vaginal epithelial height *1000 ppm:* • No effect on uterine weight, • ↑ Uterine epithelial proliferation • ↑ Vaginal epithelial height	
	Estradiol (SC injection)	1.5 µg/kg/day to PND 30 for 12 days	5	Uterotrophic response	~5.8-fold increase	

DES = Diethylstilbestrol; E2 = Estradiol.

Equol

Based on *in vitro* studies, equol is predicted to act as a weak estrogen with a potency similar to or somewhat less than genistein. Overall, equol is considered "positive" in *in vivo* studies using classic measures of estrogenicity, although a number of studies suggest that equol may not be exerting these effects with a potency predicted from the *in vitro* studies. Data presented in Allred *et al.*, 2005 *(225)*, discussed below, show that a smaller percentage of equol is circulating in the unconjugated form compared to genistein following oral exposure and the authors suggest this may account for its reduced *in vivo* potency relative to in *vitro* predictions.

Medlock *et al.*, 1995 *(338)* compared uterotrophic dose-response in neonatal Sprague Dawley rats from the NCTR breeding colony following sc injection with equol (1, 10, 100, or 1000 µg/pup) or DES (0.001, 0.01, 0.1, 10, or 100 µg/pup) from PND 1-5. Based on uterine wet weight on PND 5, equol did not cause significant uterine weight gains at any dose level although there was a significant dry weight increase at the 1000 µg/pup dose level. DES caused a significant increase in uterine wet or dry weight at 0.01 µg/pup. The potency of equol compared to DES was calculated as 0.00001, or 10^{-5}. Equol also caused significant decreases in uterine gland number/uterine section at 100 and 1000 µg/pup and had no effect on luminal epithelial hypertrophy at any dose level. The combination of these findings, plus the lack of uterotrophic response, led the authors to conclude that the *in vivo* effects of equol were not consistent with it acting as either an estrogen or anti-estrogen.

Nielsen *et al.*, 2009 *(339)* compared the estrogenicity of cow's milk containing "low" (57.4 ng/ml **[0.24 µM]**, sum of free and unconjugated equol) or "high" (1003 ng/ml **[4.14 µM]**) equol content in immature female NMRI mice treated for a period of 7-days. **[This study is also described in more detail in Chapter 3a.]** The total phytoestrogen content of the of the "high" equol milk was ~ 10-times higher than the "low" equol milk, 1215 ng/ml verus 118 ng/ml. The content of the endogenous hormones were similar between the "high" and "low" equol milk samples (estrone=503 pg/ml versus 566 pg/ml, and 17β estradiol=60.0 pg/ml versus 64.6 pg/ml). In addition, Nielsen *et al.*, 2009 assessed the uterotrophic activity of 50,000 ng/ml equol administered in drinking water that also contained 1% Tween 80. None of the "low" equol, "high" equol or 50,000 ng/ml equol treatments resulted in an uterotropic response while a uterotrophic response was observed in the 33 ng/ml and 100 ng/ml E2 positive control groups. The mRNA expression of 6 estrogen-sensitive genes was also measured in the uterus following ingestion of "low" equol or "high" equol milk: ERα, ERβ, complement component (C3), clusterin, androgen receptor, and progesterone receptor. The expression of ERβ mRNA was significantly decreased in the "high" equol milk compared to controls or the "low" equol milk group, but there were no other differences in mRNA expression. **[It is unclear if the mRNA changes were reflected in differences in protein levels because the latter was not assessed.]** The *in vitro* estrogenic activity of the "low" equol and "high" equol milk samples and purified equol were tested in an ER reporter gene assay with MVLN cells (MCF-7 human breast carcinoma cells that have been stably transfected with an estrogen receptor controlled luciferase reporter gene). Overall, there was a dose-dependent increase in the estrogenicity of equol. The author's conclusions was that the higher *in vitro* estrogenicity of "high" equol milk was not reflected as increased uterine weight *in vivo* although the down-regulation of ERβ in the uterine tissue could suggest estrogenic activity at the gene expression level.

Rachon *et al.*, 2007 *(340)* reported that uterine weights in ovariectomized Sprague-Dawley rats fed a dietary dose of 400 mg/kg equol for 6-7 weeks were significantly increased compared to control

values (114 mg versus 88 mg). The magnitude of the effect was 3.5 times lower than the uterotrophic response observed after rats ingested 10 mg/kg of estradiol-3 benzoate. The authors noted that the 400 mg/kg dose of equol was selected because previous experiments showed that lower doses did not exhibit estrogenic activity on the uterus.

Selvaraj *et al.*, 2004 *(341)* evaluated the effects of dietary (500 or 1000 ppm) or sc injection (4, 8, 12, or 20 mg/kg/day) administration of equol on uterine and vaginal endpoints in ovariectomized C57Bl/6 mice fed a phytoestrogen-free diet. The total serum levels of equol measured 2-hours following the last injection were: 4 mg/kg/d (1.4 μM), 8 mg/kg/d (2.6 μM), 12 mg/kg/d (5.1 μM), and 20 mg/kg/d (7.5 μM). The total serum levels of equol at the 12 and 20 mg/kg/day were similar to the total serum levels of equol (measured at lights on) in animals fed dietary concentrations of 500 ppm (5.9 μM) or 1000 ppm (8.1 μM). Despite the similarity in total serum equol concentrations, equol was uterotrophic following sc injection of 12 and 20 mg/kg/day, but not following dietary ingestion of 500 or 1000 ppm for 12 days. Equol was considered to be weakly estrogenic following dietary ingestion based on increased uterine epithelial Ki-67 labeling at 1000 ppm and increased vaginal epithelial height at 500 and 1000 ppm. These same effects were observed at 8, 12, and 20 mg/kg/day. The authors attributed this difference in potency based on route of administration to the impact of phase II conjugation of equol in the gastrointestinal tract following dietary ingestion. The percentage of total equol present as the aglycone following sc injection treatment ranged from 17–20%, depending on administered dose, compared to 2% in animals treated via the diet.

Bateman and Patisaul, 2009 *(317)* compared the day of vaginal opening and regularity of the estrous cycle following neonatal sc injection treatment with estradiol benzoate (50μg), genistein (10 mg/kg), or racemic equol (10 mg/kg) beginning on the day of birth (PND 0) once daily through PND 3 (total of four injections). **[This study is described in more detail in Chapter 3a, including discussion of other endpoints related to GnRH and kisspeptin fiber density in the hypothalamus.]** On PND 22 the pups were weaned and examined daily for vaginal opening, monitoring of estrous cycle began two weeks later and continued for 12-13 weeks. Vaginal opening was significantly advanced by ~5 days in the estradiol benzoate group and ~1.5 days in the genistein group (n=8) but not in the equol group (n=10) compared to controls (n=12). Regular 4-day estrous cycles commenced in all groups, but all the EB females (n=10) stopped cycling and entered persistent estrus within 3 weeks. By 10 weeks, less than 30% of the equol (n=8) and the genistein (n=7) treated females displayed regular estrus cycles; the remainder were either in persistent estrus or diestrus. The rate at which genistein and equol treated animals became acyclic was very similar.

Brienholt *et al.*, 2000 *(342)* compared the uterotropic activity of 100 mg/kg genistein or equol in mice treated during PND 17-20 by gavage. Both compounds caused significant increases in uterine weight (corrected for body weight), although the magnitude of the effect was greater for genistein (3-fold increase compared to controls) than for equol (2-fold increase compared to controls).

The studies described above provide some basis for comparing the *in vivo* estrogenic activity of genistein, daidzein, and equol using the typical endpoints of uterotrophic response, onset of sexual maturity, or estrous cyclicity. In addition to these studies, a number of investigators have drawn conclusions on the relative *in vivo* potency of these isoflavones based on animal models of mammary gland carcinogenesis that include use of xenografts of human breast cancer cell lines grown in immune-deficient mice and chemically-induced (*e.g.,* 7,12-dimethylbenzanthracene; N-nitrosomethylurea) models in rats or mice.

The interpretation from studies conducted by Allred *et al.*, 2004, 2005 was that genistein is primarily responsible for the estrogenic acitivity of isoflavone mixtures administered to mice. In the first study, Allred *et al.*, 2004 *(344)* compared the degree of stimulation of human MCF-7 tumor growth in ovariectomized athymic mice fed 5 different diets that provided the same amounts of genistein aglycone equivalents, ~750 µmol/g, but differed in the degree of processing. The relative ranking of the 5 diets in terms of processing was soy flour supplemented with a mixture of isoflavones < soy molasses, an alcohol extract of soy flour < Novasoy, a product containing 40% isoflavones made by removing the carbohydrates from soy molasses < an isoflavone mixture produced by crystallization of Novasoy < purified genistin. During an 11-week period, the average tumor surface area increased more in animals fed the more processed diets. While tumors in the negative control animals regressed throughout the study, the tumors in the soy flour-fed animals were relatively stable in size (neither grew nor regressed). Tumor growth was stimulated in animals fed soy molasses, Novasoy, mixed isoflavones or genistin alone compared with animals consuming a control diet devoid of soy.

In 2005, Allred *et al.(225)* published a follow-up study that compared the plasma pharmacokinetics and metabolite profile in mice fed the 5 diets described above to determine whether differences in those variables could account for the differences in tumor stimulating potential. The degree of processing did affect a number of variables related to bioavailability and metabolism of daidzein to equol. However, only plasma concentrations of genistein aglycone correlated significantly with stimulation of tumor growth. **[The overall trend was that concentrations of genistein aglycone, but not total genistein, increased with greater degrees of isoflavone processing. A significant inverse correlation was observed between total or aglycone equol and tumor growth.]** In addition, analyses of the total isoflavones present as aglycone, sulfate, or glucuronides in mice fed the soy flour diet showed that the fraction of equol as aglycone was much lower when compared to genistein aglycone or daidzein aglycone (**Table 72**). The author's conclusion on the relative biological activity of genistein and equol was that equol may be less potent *in vivo* compared to genistein than would be predicted from *in vitro* estimates because a much lower fraction of equol is circulating as the biologically active aglycone, 1% versus 9%, (**Table 72**). The authors did not consider it biologically plausible that equol was antagonizing the stimulatory estrogenic effects of genistein on MCF-7 tumor growth because of the *in vitro* evidence that equol is acting as estrogen agonist and not as an estrogen antagonist.

Other work from this research group was also interpreted as indicating that genistein is the major active estrogenic component of soy isoflavones. Ju *et al.*, 2006 *(345)* evaluated the potential of daidzein and (±)-equol in stimulating MCF-7 cell growth *in vitro* and *in vivo* following implantation into ovariectomized athymic mice. Both daidzein and (±)-equol stimulated MCF-7 cell proliferation *in vitro* at concentrations between 0.001 and 50 µM, with maximal stimulation at 1 µM. For the *in vivo* assessment, tumor size, proliferation, and pS2 expression were measured in athymic mice that were fed

Table 72. Distribution of Isoflavones and Equol in Mice Fed a Soy Flour Diet (Allred et al., 2005)

Form	*Genistein (%)*	*Daidzein (%)*	*Equol (%)*
Aglycone	9	23	1
Sulfate	38	33	78
Glucuronides	53	44	21

From Allred et al., 2005 *(225)*

a diet with daidzein for 21 weeks (125, 250, 500, or 1000 ppm) or (±)-equol for 37 weeks (250, 500, 1000 ppm) following implantation of MCF-7 cells. Dietary daidzein had a slight but significant effect on stimulating MCF-7 xenografts in mice but no effect on pS2 mRNA induction. Equol production was variable across groups in the daidzein-fed mice and ranged from 0 to 33%, 125 ppm (2/6), 250 ppm (1/8), 500 ppm (1/7), and 1000 ppm (0/8). Equol did not stimulate tumor growth, cell proliferation in tumors, or induce pS2 expression. The plasma levels of daidzein and (±)-equol in the mice were similar to the concentrations that stimulated MCF-7 proliferation *in vitro*. The overall conclusions of the authors based on this study and their prior work was that daidzein and equol have only minimal estrogenic effects *in vivo* and that genistein is the major active estrogenic component of soy isoflavones.

Ju *et al.*, 2006 considered these findings consistent with the results of Fritz *et al.*, 1998 *(209)* and Lamartiniere *et al.*, 2002 *(346)* showing that (1) prepubertal administration of a diet containing 250 mg/kg daidzein did not affect mammary gland differentiation or reduce the ontogeny of chemically induced mammary tumors following treatment with DMBA, and (2) similar treatment with genistein caused a reduction in the numbers of undifferentiated mammary gland structures, i.e., terminal end buds, and reduced tumor multiplicity in response to DMBA treatment. In the daidzein feeding study, the concentrations of equol in the mammary tissue was 17% of the level measured in blood and the aglycone was not detected in the mammary gland tissue. For genistein, mammary tissue levels were 61% of the blood level and 72% of genistein measured in the mammary tissue was present as the aglycone.

2.2.9.3 In vitro

In vitro estrogenicity assays are not necessarily predictive of *in vivo* effects *(3)*. These assays do not account for *in vivo* processes such as absorption, distribution, binding to serum proteins, and metabolism. Whereas *in vivo* assays allow for the evaluation of the total response resulting from direct and indirect mechanisms of toxicity, *in vitro* assays allow for only responses occurring through an individual type of ER system under study. Results in test systems utilizing yeast cells or mammalian cell cultures can be affected by kinetics or membrane transport activities that have no relevancy to *in vivo* exposures; for example, yeast cells have the ability to eliminate certain types of compounds.

The summary of *in vitro* measurement of estrogenicity (**Table 73**) is based primarily on reviews by Whitten and Patisaul *(108)* and Chen and Rogan *(13)*, with the inclusion of additional studies that were not addressed in the reviews. The results are expressed as relative potency, most often in comparison to 17β-estradiol. Potency of the compounds was found to vary across assays, possibly as a result of different experimental protocols and variations in ER subtypes *(3)*. However, the results consistently demonstrate that genistein, daidzein, and equol weakly induce estrogenic activity, with potencies well below that of 17β-estradiol. Kurzer and Xu *(179)* theorized that genistein, daidzein, and equol at high doses could potentially act as antiestrogens by competitively binding to ERs, thus preventing binding of endogenous estrogens.

Utility of *in vitro* assays is also affected by the type of ER expressed. Two main types of ERs identified to date are ERα and ERβ. Compounds differ in their relative binding affinities for the two ER subtypes, and it appears that most phytoestrogens bind preferentially to ERβ. Distribution of the two receptor subtypes varies according to estrogen-responsive tissue and developmental stage. Expression of only the ERα subtype in most assay systems limits usefulness of *in vitro* assays for predicting *in vivo* estrogenicity responses *(108)*. Assays that do not include significant levels of ERβ are likely to

Table 73. In Vitro Estrogenicity of Genistein, Daidzein, and Equol

Model	*Percent 17β – Estradiol Potency*		
	Genistein	*Daidzein*	*Equol*
ER Mediated Events			
Uterine cytosol from Sprague Dawley rats fed phytoestrogen-free diet[a]	1		
Uterine cytosol from rat or sheep[b,c]	0.45 – 2	0.023 – 0.1	0.2 – 0.4
ERs from mouse uterine cytosol[d]	0.87	0.082	
Liver cytosol[b]	0.1	0.01	
MCF_7 breast cancer cells[b]	0.1 – 2		0.1
hER-transfected yeast[b]	0.05		
hER-transfected COS7 cells[b]		0.01	
Synthesized human ERα protein[b,c]	5		
Synthesized rat ERβ protein[b,c]	36		
Human ERα-transfected baculovirus – Sf9 insect cell system[c,e]	0.7	0.2	
hERβ-transfected baculovirus – Sf9 insect cell system[c,e]	13	1	
Estrogen-dependent pituitary tumor cells[f]	0.88 relative to diethylstilbestrol		
ER Reporter Assays			
pS2 (estrogen-regulated gene response) in MCF_7 breast cancer cells[b,c]	0.001 – 0.1		
Exoprotein: MCF_7 breast cancer cells[b]	0.01	0.002	
Alkaline phosphatase activity in Ishikawa-Var I human endometrial adenocarcinoma cells[g]	0.084	0.013	0.061
BG1Luc4E2 cell line[c]	0.001	0.0004	
Human ER-galactosidase reporter-transfected yeast[b]	0.01 – 0.05	0.001	0.085
Human ERα-*LacZ* reporter-transfected yeast[i]	0.02 – 0.044	0.00045	
hER-Chloramphenicol acetyltransferase reporter-transfected Le42	0.04	0.003	
TATA-Luciferase-reporter transfected T47D human breast adeno-carcinoma cells[h]	0.006		
Human ERα-TATA-luciferase reporter-transfected human embryonal kidney 293 cells[c]	0.025		
Human ERβ-TATA- luciferase reporter-transfected human embryonal kidney 293 cells[c]	0.8		
ERα-luciferase reporter-transfected HepG2 human hepatoma cells[c]	1	0.08	
ERβ-luciferase reporter-transfected HepG2 human hepatoma cells[c]	30	1.7	
Cell proliferation			
MCF_7 breast cancer cells[b]	0.01 – 0.08	0.0007	

[The use of 17β-estradiol as a reference compound was not always explicit.]

[a] Santell et al., 1997 *(211)*.
[b] Reviewed in Whitten and Patisaul, 2001 *(108)*.
[c] Reviewed in Chen and Rogan, 2004 *(13)*.
[d] Zhang et al., 1999 *(248)*.
[e] Kuiper et al., 1998 *(347)*.
[f] Stahl et al., 1998 *(348)*.
[g] Markiewicz et al., 1993 *(349)*.
[h] Legler et al., 1999 *(350)*.
[i] Dhooge et al., 2006 *(351)*

underestimate estrogenic response to genistein. In addition, recombinant cells can be more sensitive to estrogenic effects because they often contain multiple copies of estrogen response elements. Studies that use a positive control such as E2 or DES can provide relative potency estimates but may not accurately predict concentrations of compounds required to induce effects *in vivo*.

A number of publications have compared the estrogenicity of isoflavones using a variety of *in vitro* model systems. Breinholt and Larson, 1998 *(352)* compared the estrogenicity of genistein, daidzein, and equol based on transcriptional activity of ER in a recombinant yeast screen and in a MCF-7 cell proliferation assay (**Table 74**).

Table 74. Comparison of In Vitro Measures of Isoflavone Estrogenicity (Breinholt and Larson, 1998)

Compound	*Fold Induction*	*EC_{50} (μM)*	*% of 17β-E_2 Response*
Estrogenic activity based on yeast screen transcriptional activation			
17β-Estradiol	12.6	0.000023	100
Genistein	11.8	0.5	0.0045
Daidzein	9.4	8.5	0.00028
Equol	12.6	0.1	0.023
Estrogenic activity based on MCF-7 cell proliferation			
17β-Estradiol	8.1	0.000077	100
Genistein	5.8	0.3	0.026
Daidzein	3.8	0.7	0.011
Equol	5.5	0.6	0.013

From Breinholt and Larson, 1998 *(352)*.

In a study by Choi *et al.*, 2008 *(141)*, the estrogenic activities of genistein, daidzein, glycitein, and equol were compared to to E_2 using ERα and ERβ binding, a yeast transcriptional activation assay with, and cell proliferation in MCF-7 cells ("E-screen"). The overall relative potency ranking for the isoflavones was genistein > equol > daidzein ≈ glycitein (**Table 75**).

Table 75. Comparison of In Vitro Measures of Isoflavone Estrogenicity (Choi et al., 2008)

Compound	*Relative Binding Affinity (%)*[a]			*Relative Estrogenic Activities*		
	ERα	*ERβ*	*β/α*	*ER Binding*[b]	*Yeast Transactivation*[b]	*E-Screen*[b]
Estradiol	100	100	1	++++	++++	++++
Genistein	2.07	14.8	7.1	+++	++++	+++
Daidzein	0.55	0.46	0.8	++	++	++
Equol	1.70	4.45	2.6	++	+++	++++
Glycitein	0.32	0.44	1.4	++	N.D.	++

N.D. = Not determined.

[a] Relative binding affinity = (IC50 of E2) ÷ (IC50 of test compound) × 100.

[b] Based on comparisons to estradiol alone: ++++ (≥100%), +++ (66%–99%), ++ (33%–66%), + (1%–33%). Potency estimates for ER binding were based on binding data for at least one ER type.

Modified from Table 1 in Choi et al., 2008 *(141)*.

Genistein displays preferential binding to ERβ compared to ERα *(353; 354)*. For example, based on ligand binding analysis of *in vitro* synthesized human ERα and rat ERβ, Kuiper *et al.*, *(354)* reported that the relative binding affinity (RBA) of genistein was approximately 7-times higher for ERβ compared to ERα. Similarly, using HELN-ERα or HELN-ERβ responsive reporter cell lines, Escande *et al.*, *(355)* reported that the ratio of relative binding affinity for ERβ to ERα for genistein was ~ 27. Other *in vitro* studies suggest differences in genistein activation of ERα and ERβ at low and high concentrations. Chang *et al.*, 2008 *(356)* reported that a low concentration of genistein (6 nM) regulated gene expression preferentially through ERβ; however, at a high concentration of genistein (300 nM) gene expression changes were nearly identical to estradiol in cells expressing ERα alone or both ERα and ERβ, suggesting the activation of both ER subtypes.

Muthyala *et al.*, 2004 *(357)* compared the binding and transcriptional activation through ERα and ERβ for genistein, daidzein, racemic (±)-equol, the naturally occurring *S*-equol enantiomer, and the unnatural *R*-equol enantiomer (**Table 76**). All of the equol isomers displayed higher affinity for ERα and ERβ compared to daidzein. Genistein displayed a much higher binding affinity for ERβ than daidzein. The absolute binding affinities (*Ki* values) on ERα and ERβ differed between *S*-equol (β/α=13) and *R*-equol (β/α=0.29) such that *S*-equol exhibited a high ERβ preference. The binding affinity for racemic (±)-equol was almost the average of the individual equol enantiomers. Based on the transcriptional activation curves, all of the compounds were agonists for ERα and ERβ and gave maximal efficacies of reporter activity that were similar to estradiol although genistein was considered somewhat of a "superagonist" for ERα and the maximum efficacy of *R*-equol was slightly less than that of estradiol. **[The Expert Panel notes the term "superagonist" may not be appropriate, especially given that E2 has a binding affinity in the nM range while genistein has a binding affinity in the µM range. Studies of specific receptor binding and displacement coupled with studies with specific reporters/ gene expression would need to be conducted to demonstrate superagonism.]**

Table 76. Comparison of Binding Affinities and Reporter Gene Activation for Isoflavones and Equol for ERα and ERβ (Muthyala et al., 2004)

Compound	RBA (%)			K_i (nM)			EC50 (nM)[a]		
	hERα	hERβ	β/α	hERα	hERβ	β/α	hERα	hERβ	β/α
Estradiol	100	100	1	0.2	0.5	0.4	0.021	0.11	0.19
Daidzein	0.010	0.040	4	2000	1300	1.5	250	100	2.5
Genistein	0.017	7.4	440	1200	6.7	180	80	6.6	12
(±) Equol	0.20	1.60	8	100	31	3.2	200	74	2.7
R(+) Equol	0.40	0.30	0.7	50	170	0.29	66	330	0.20
S (–) Equol	0.10	3.20	32	200	16	13	85	65	1.3

[a] Transcriptional activation measured using a co-transfection assay in human endometrial carcinoma cells-1 (HEC-1).
RBA=relative binding affinity; K*i*=equilibrium binding competition constant: EC50=concentration producing response halfway between baseline and maximal response.
From Muthyala et al., 2004 *(357)*.

Assessment of relative estrogenicity can also vary as a function of cell system used. For example, Buterin *et al.*, 2006 *(358)* compared the gene expression profiles of 1 µM genistein and 30 pM E2 in MCF-7 and T47D human carcinoma cell lines. A fraction of RNA from each sample was

analyzed using Affymetrix microarrays that display sequences of 33,000 human transcripts; ~35% of the surveyed gene products were present at detectable levels based on use of Microarray Suite version 5.0 software. The authors used DNA-Chip Analyzer (dChip) software to identify transcripts that were susceptible to ER regulation where hybridization data was filtered using a cutoff of >2.5 fold change in a least one treatment groups. The number of genes whose transcription considered susceptible to estrogenic stimulation was higher in the MCF-7 cells (n=134) compared to T47D cells (n=76). The genomic signature of genistein in MCF-7 cells was considered to be very similar to that of a physiologically relevant concentration of E2. Based on linear regression, the correlation in MCF-7 cells between genistein and E2 for 134 transcripts considered to be estrogen-dependent was 0.978.

Glucuronidation and sulfation of isoflavones produce structural changes that can lower receptor binding affinity. One *in vitro* study reported that the conjugated forms of both genistein and daidzein have lower receptor affinity than their parent compounds. Zhang *et al.*, 1999 *(157; 197)* characterized the relative affinities of genistein, genistein glucuronide, daidzein, and daidzein glucuronide for $B6D2F_2$ mouse uterine cytosolic estrogen receptors. The glucuronides displayed lower relative binding affinities Genistein exhibited weak ER binding compared to 17β-estradiol (**Table 77**). With a relative binding affinity of 0.02, genistein glucuronide also displayed a weak affinity for the ER that was less than the affinity of the aglycone (0.87). **[Given the relative tissue distributions of ERα and ERβ in the uterus, uterine binding is probably an assessment of ERα binding]**. No hydrolysis by uterine cytosol was observed **[data were not shown]**.

Table 77. Relative Affinities of 17β-Estradiol, Diethylstilbestrol, and Isoflavone Glucuronides for Mouse Uterine Cytosolic Estrogen Receptors (Zhang et al., 1999)

Compounds	*Relative Affinity*
17β-estradiol	100
Diethylstilbestrol	91.8
Genistein	0.87
Genistein glucuronide	0.018
Daidzein	0.082
Daidzein glucuronide	0.009

From Zhang et al., 1999 *(248)*.

Several *in vitro* studies suggest that genistein may affect the activity of enzymes involved in E2 metabolism and elimination, potentially impacting the bioavailability of endogenous estrogens. Harrington *et al.*, 2006 *(359)* reported that UGT2B15 mRNA levels are up-regulated by estradiol and genistein (1 μM) in MCF-7 cells. This particular enzyme was reported to be the only UGT2B enzyme up-regulated by estrogens and the authors suggested its up-regulation may modulate local concentrations of estrogens and androgens and their signaling in breast cancer cells. Hanet *et al.*, 2008 *(360)* reported that genistein inhibited mRNA and protein expression of two phase II enzymes involved in forming conjugated E2 metabolites, UGT1A1 and SULT1E1, in the ER-negative hepatoblastoma HepG2 cell lines, indicating that the effects were not mediated by ER binding. Pretreatment of cells with genistein led to intracellular retention of $[^3H]$-E_2, an effect the authors suggest is more likely a result of decreased efflux rather than increased influx of $[^3H]$-E_2.

2.2.10 Non-Estrogenic Mechanisms of Action

In addition to ER-mediated activity, genistein and other isoflavones have other activities including acting as inhibitors of aromatase *(361)*, tyrosine kinase and topoisomerase *(362)*(reviewed in *(179; 363)*, inducers of Phase I and/or Phase II enymes *(188; 364)*, and cause effects on cell cycle control *(365)*. Tissue or organ systems implicated by these non-ER pathways include bone, adipose tissue, and the cardiovascular system and processes related to inflammation, oxidative stress, and angiogenesis *(366)*. Most studies that compare the relative activities of genistein, daidzein, or equol and relatively few include assessment of glycitein. For some measures glycitein is the more potent isoflavone. For example, Clubbs and Bomser, 2007 *(367)* reported that glycitein was a more potent activator of extracellular signal-regulated kinase (ERK1/2) activity in a non-tumorigenic prostate epithelial cell line (RWPE-1). Glycitein activation was dependent on vascular endothelial growth factor receptor (VEGFR) signaling and resulted in a decrease of RWPE-1 cell proliferation by 40%, a finding the authors suggest may contribute to an anticancer activity of soy in the prostate.

Some of these mechanisms have been implicated as pathways through which genistein and other isoflavones could act to inhibit carcinogenesis. Genistein stimulates *in vitro* cell proliferation at concentrations <10 μM **[2700 μg/L] [agonist activity]** but inhibits proliferation at concentrations >10 μM **[2700 μg/L] [antagonist activity]** (reviewed in *(108)*). Stimulation of cell proliferation at low doses is thought to result from estrogenic activity. Possible mechanisms for suppressed cell proliferation at higher genistein doses include inhibition of protein tyrosine kinases and DNA topoisomerases (reviewed in *(179; 363)*). Tyrosine kinases are oncogene products thought to induce cell proliferation through phosphorylation of tyrosine residues of growth factors associated with tumor cell signal transduction and proliferation pathways. DNA topoisomerases catalyze configurational changes in DNA. There is evidence that tyrosine kinase or topoisomerase inhibition can result in suppression of angiogenesis (reviewed in *(179)*). Studies in three cancer cells lines suggested that genistein stabilizes the normally transient bond between DNA and topoisomerase II, resulting in double strand breaks. The DNA breaks can lead to altered gene expression or terminal cellular differentiation, processes that inhibit cancer cell proliferation (reviewed in *(363)*). Apoptosis is another possible consequence resulting from genistein-induced topoisomerase inhibition and resulting DNA breaks (reviewed in *(3; 363)*). Genistein-induced inhibition of protein tyrosine kinase (≥2.6 μM **[700 μg/L]**) and DNA topoisomerases II activity (≥4 μM **[1080 μg/L]**) was demonstrated in numerous cancer cell lines *(3; 108; 179)*.

Reactive oxygen species can damage DNA, cellular proteins, and lipids and may be involved in carcinogenesis *(3)*. Antioxidant activity of genistein was demonstrated in *in vitro* and *in vivo* studies. In *in vitro* assays, genistein inhibited the generation of superoxide and hydrogen peroxide radicals at concentrations ≥1 μM **[270 μg/L]** *(3; 108; 179)*. Other *in vitro* assays demonstrated that genistein reduced free-radical-induced DNA damage, lipid peroxidation, and low-density lipoprotein oxidation at concentrations ≥10 μM **[2.7 mg/L]** *(3)*. Antioxidant activity was also observed with daidzein and its metabolites equol and O-desmethylangolensin. In experimental animals dosed with genistein or soybean isoflavone extracts, there was an increase in antioxidant enzyme activities, specifically activities of catalase, superoxide dismutase, glutathione peroxidase, and glutathione reductase in skin and small intestine of mice, and activity of cumene hydroperoxidase in rat liver (reviewed in *(179)*). **[Isoflavones are potent radical scavengers in the absence of antioxidant enzymes as well.]**

It has been postulated that soy protein isolate can reduce carcinogenicity of procarcinogens such as dimethylbenzanthracene by inhibiting CYP enzymes involved in bioactivation (*(368)* reviewed

in *(33)*). Rats fed diets containing soy protein isolates compared to casein had reduced levels of CYP1A1 protein in liver and CYP1A1, CYP1A2, and CYP1B1 proteins in mammary gland. Similar reductions in CYP protein expression were observed in rats fed whey-based diets. Feeding of soy protein isolate to rats exposed to dimethylbenzanthracene lowered levels of dimethylbenzanthracene-deoxyribonucleic acid (DNA) adducts in tissues (reviewed in Badger *et al.*, 2002 *(33)*Thus, it has been suggested that down-regulation of CYP1A1 and CYP1B1 is a possible mechanism of cancer protection in dimethylbenzanthracene-treated rats. Soy protein diets treated to remove isoflavones also provided protection against dimethylbenzanthracene-induced cancer in rats, suggesting that the protective effects of soy are not due to isoflavones. Studies in humans ingesting soy products with ≥12 mg genistein or ≥56 mg isoflavones demonstrated reductions in oxidized low-density lipoprotein, lipoprotein peroxidation, or oxidative DNA base damage (reviewed in *(3)*).

Genistein is a known activator of the mutated gene that causes cystic fibrosis (reviewed in *(369-372)*. Cystic fibrosis transmembrane conductor regulator (CFTR) is an apical membrane protein that acts as a chloride channel and regulates chloride and sodium transport in secretory epithelial cells. Mutations in CFTR disrupt protein production or function by a number of molecular mechanisms and several categories have been developed to classify the mutations. Class III mutations result in a CFTR that is resistant to phosphorylation or ATP binding resulting in defective regulation. Induction of chloride transport follows phosphorylation of the regulatory domain of CFTR which causes binding of ATP to the nucleotide binding domain. Class III mutations result in a CRTF that can be produced, transported and inserted into the apical membrane but not effectively regulated. Genistein can directly activate mutant and wild-type CFTR. For example, it can overcome the affected ATP binding for the class III mutation G551D, possibly by directly binding to the nucleotide binding domain of a phosphorylated CFTR (reviewed in *(371)*). Genistein's role as a CFTR activator is being considered therapeutically to increase chloride conductance of mutated CFTR that is available in the cell membrane.

In vitro studies show that genistein is an agonist for the estrogen-related receptor ERRα *(373; 374)*; reviewed in *(375)*). ERRs (ERRα, ERRβ, and ERRγ) are orphan nuclear receptors that may modulate estrogen responsiveness by binding to estrogen response elements. Although the DNA-binding domain of ERRs are similar to those of ERα and ERβ, the ligand binding domains are less similar and ERRs do not bind endogenous estrogens. In addition, the ERRs are constitutively active wherease ERs are ligand activated. Genistein has also been shown to have a high binding affinity for a GPR30, a recently identified seven-transmembrane ER with a relative binding affinity that is 13% of 17β-estradiol *(376)*. GPR30 is not well-characterized but reportedly responds to estrogens with rapid signaling that includes ERK activation, PI3K activation, calcium mobilization and cAMP production *(377)* and is being investigated for its potential role in regulating estrogen-associated responses, including vascular signaling pathways and breast cancer progression *(378; 379)*.

Genistein and daidzein can also bind and initiate transcriptional activation of the peroxisome proliferation-activated receptor gamma (PPARγ). PPARγ is a ligand-dependent transcription factor of the nuclear hormone receptor superfamily involved in regulating adipogenesis and metabolism of glucose and cholesterol. Dang *et al.*, 2003 *(380)* reported that genistein at high concentrations *in vitro* (> 1μM) acts as a ligand for PPARγ, leading to the upregulation of adipogenesis and down regulation of osteogenesis. In contrast, at lower concentrations *in vitro*, genistein acts as an estrogen and exerts opposite effects on adipogenesis and osteogenesis.

In general, the relative potency for activating ER-dependent activities associated with soy isoflavones is: genistein ≥ equol > daidzein; this potency ranking is primarily based on data from *in vitro* reporter assays and relative receptor binding affinity *(3)*. Studies, mostly *in vitro*, have also examined effects of soy isoflavones on other endpoints, such as effects on bone, cardiovascular/lipid regulation, cell growth, inflammation, immunity and neurology (**Table 78**). Of the 77 studies that presented data on these endpoints, the majority reported a similar pattern of relative ranking for genistein, equol and daidzen (i.e., magnitude of effect or relative potency). Across these studies, genistein was more potent than equol or daidzen in 60 of approximately 117 endpoints examined. The relative effect for all three isoflavones were similar in another 52 of these endpoints. Daidzein or equol caused a greater effect as compared to genistein for only five endpoints. It is worth noting that 16 of these studies also reported that genistein inhibited tyrosine kinase activity, while inhibition of this enzyme by daidzein was not observed. The tyrosine kinase activity data suggest that the effects of genistein could be due in part to a non-ER mode of action. In all cases where an effect was observed, the isoflavones act in the same direction (e.g., genistein and daidzen both inhibited bone resorption *(381)*). Collectively, these data do not support the notion that daidzein or equol markedly "offset" genistein activity.

2.3 Genetic Toxicity

Results of *in vitro* genetic toxicity testing for genistein are listed in **Table 79**. With the exception of one weak positive result in one strain of *Salmonella* following metabolic activation, bacterial tests did not indicate mutagenicity. Positive results were generally observed in mutation, micronuclei, chromosomal aberration, and deoxyribonucleic acid (DNA) strand break tests conducted in mammalian cells; only one of the assays utilized metabolic activation. Results of *in vivo* micronuclei tests (4 in mice and 3 in rats), 1 chromosomal aberration test in mice, and 1 sister chromatid exchange test in mice are summarized in **Table 80**. In contrast to the *in vitro* tests, the *in vivo* tests did not suggest that genistein induced micronuclei or chromosomal aberrations. No genotoxicity studies of soy formula were identified.

Bennetts *et al.*, 2008 *(458)* evaluated a number of estrogenic compounds, including genistein, for their ability to create oxidative stress and DNA damage in spermatozoa. Genistein was only active in stimulating the generation of reactive oxygen species at the highest dose tested, 500 µM, and essentially considered inactive in creating oxidative stress. No data was reported related to genistein and DNA damage. Ullah *et al.*, 2009 *(459)*, using the Comet assay, subsequently reported that both genistein and its methylated analog, biochanin A, induce DNA strand breaks in cultured human lymphocytes at concentrations of 10 to 50 µM. They present evidence that the DNA damage is the result of the reactive oxygen species, specifically superoxide anions and hydroxyl radicals. Pugalendhi *et al.*, 2009 *(460)* investigated the antigenotoxic effects of genistein by pre-treating female rats orally with 20 mg/kg bw/day genistein for 5 days, followed by intraperitoneal injection of 30 mg/kg body weight DMBA.They reported that DMBA treatment alone induces significantly high levels of micronuclei and chromosomal aberrations in bone marrow cells and that pre-treatment with genistein significantly reduces these effects.Genetic toxicity information for daidzein and its metabolites and for glycitein is more limited than for genistein. In a review by Kulling *et al.*, 2002 *(461)* it is noted that in V79 cells, daidzein is negative for all endpoints tested including micronuclei, HPRT mutations, mitotic arrest, and abnormal mitotic spindles, and that negative results were also found in a chromosomal aberration test using cultured human lymphocytes. In a more recent review, Stopper *et al.*, 2005 *(462)* note the following. Daidzein, tested up to its limit of solubility, is negative in two micronucleus studies using V79 and L5178Y cells. Another study found a slight increase in micronuclei using V79 cells.

Table 78. Non-Estrogenic* In Vitro *and* In Vivo *Relative Activities of Genistein, Daidzein and Equol

Model	*Relative Activity*			*Reference*
	Genistein	*Daidzein*	*Equol or Glycitein*	
Skeletal System				
Bone resorption in avian osteoclasts*	↓(IC50 μM)	↓(30 μM, highest concentration tested)	–	Blair et al., 1996 *(381)*
Bone attachment in avian osteoclasts*	No effect (3–30 μM tested)	No effect (10 μM tested)	–	
Glycosaminoglycan (GAG) synthesis in female bovine articular chondrocytes	↓(10–5 M) **[≥10 μM]**	↓(10–5 M) **[≥10 μM]**	–	Classen et al., 2008 *(382)*
Insulin-stimulated sulfate uptake in female bovine articular chondrocytes	↑(≥10–9 M)[a] **[≥0.000001 μM]**[a]	↑(≥10–11 M)[a] **[≥0.00000001 μM]**[a]	–	
Calcium content, DNA content in cultured rat femoral-diaphyseal tissues	↑(≥10–7 M) **[≥0.1 μM]**	↑(≥10–7 M) **[≥0.1 μM]**	–	Gao et al., 1999 *(383)*
Alkaline phosphatase activity in cultured rat femoral-diaphyseal tissues	↑(≥10–6 M) **[≥1 μM]**	↑(≥10–6 M) **[≥1 μM]**	–	
Cell proliferation of MC3T3-E1 cells, a mouse calvaria osteoblast-like cell line	No effect (0.01–10 μM tested)	No effect (0.01–10 μM tested)	–	Kanno et al., 2004 *(384)*
Calcium and phosphorous content in MC3T3-E1 cells	↑(10–6 M, highest concentration tested) **[1 μM]**	↑(10–6 M, highest concentration tested) **[1 μM]**	–	
Effect on bone mineral density (lumbar vertebrae, total femur, distal femur metaphysis, femoral diaphysis) ovariectomy-induced bone loss in rats after 3 months of dietary ingestion of 10 mg/kg/day of genistein or daidzein	No effect (10 mg/kg bw/day)	↑ (10 mg/kg bw/day)	–	Picherit et al., 2000 *(385)*
Protein content and alkaline phosphatase activity in osteoblastic MC3T3-E1 cells	↑(10–6 M) **[≥1 μM]**	↑(10–6 M) **[≥1 μM]**	–	Sugimoto et al., 2000 *(386)*
Protein synthesis in osteoblastic MC3T3 cells	↑(≥0.1 μM)	↑(≥0.1 μM)	–	Yamaguchi et al., 2000 *(387)*

Table 78 (continued)

Model	Relative Activity			Reference
	Genistein	Daidzein	Equol or Glycitein	
Cardiovascular System/Lipid Regulation				
Impact on strength of histamine-induced muscle contraction in guinea pig left atrium*	Attenuated (50 μM)	No effect (50 μM)	–	Akaishi et al., 2000 *(388)*
Hepatocyte cholesterol metabolism and apolipoprotein (apo) B secretion in human hepatoma cells (HepG2), relative to control response				Borradaile et al., 2002 *(389)*
ApoB secretion	↓69.5% (50 μM)	↓71% (50 μM)	–	
Activity of microsomal triacylglycerol transfer protein (MTP)	↓76% (50 μM)	↓86.5 (50 μM)	–	
LDL receptor mRNA	↑3.1-fold (50 μM)	↑2.3-fold (50 μM)	–	
LDL receptor binding, uptake, and degradation	↑~125–162% (50 μM)	↑~125–155% (50 μM)	–	
Microsomal activity of ACAT1 and ACAT2	↓74.8–75% (50 μM)	↓88–91.9% (50 μM)	–	
Activation of CFTR chloride current in guinea pig ventricular myocytes*	↑(50 μM)	No effect (50 μM)	–	Chiang et al., 1997 *(390)*
Neointima formation in endothelium-denuded female rabbit aorta	↓(185 μM)	↓(197 μM)	–	Finking et al., 2000 *(391)*
Inhibition of collagen-induced human platelet aggregation	IC50=30.3 μM	IC50=38.8 μM	–	Gottstein et al., 2003 *(392)*
Inhibition of NO production in murine RAW 264×7 macrophages	IC50=57.9 μM	IC50=106.5 μM	–	
Inhibition of TNF-α secretion in murine RAW 264×7 macrophages	IC50=52.9 μM	IC50=64.2 μM	–	
Inhibition of MCP-1 secretion in human umbilical vein endothelial cells	IC50=29.4 μM	IC50=37.3 μM	–	
cAMP-regulated Cl- current in the presence of β-adrenergic agonist recorded from guinea pig ventricular myocytes	↑(50 μM)	No effect (50 μM)	–	Hool et al., 1998 *(393)*
cAMP-regulated K+ and L-type Ca2+ current recorded from guinea pig ventricular myocytes	↓(50 μM)	↓(50 μM)	–	

Table 78 (continued)

Model	Relative Activity			Reference
	Genistein	*Daidzein*	*Equol or Glycitein*	
Effect on carbachol-induced relaxation response in phenyephrine-constricted pulmonary artery rings isolated from normoxic rats and rats exposed for 14-days of hypobaric hypoxia*	↑Relaxation response in artery rings from rats exposed to hypobaric hypoxia (30 μM)	↑Relaxation response in artery rings from rats exposed to hypobaric hypoxia (30 μM)	–	Karamsetty et al., 2001 *(394)*
Apo A-1 secretion in human hepatoma cell line Hep G2	↑↑(10 μM)	↑(10 μM)	–	Lamon-Fava, 2000 *(395)*
Relaxation in rat aorta rings in pre-constricted by treatment with phenylephrine	↑(IC50=5.7 μM)	↑(IC50=36.7 μM)	–	Mishra et al., 2000 *(396)*
Percent relaxation in rat mesenteric arterial rings (endothelium denuded) following pre-constriction with noradrenaline treatment	45% (10 μM)	31% (10 μM)	–	Nevala et al., 2001 *(397)*
Growth in aortic smooth muscle cells from stroke-prone spontaneously hypertensive rats	↓(≥3 μM)	↓(≥10 μM)	↓(≥10 μM glycitein)	Pan et al., 2001 *(398)*
Effects on platelet-derived growth factor-BB induced smooth muscle cell proliferation	↓(≥0.1 μM)	↓(≥0.1 μM)	↓(≥0.1 μM glycitein)	
L-type Ca2+ membrane current in guinea pig ventricular myocytes, whole cell preparation*	↓66% (200 μM)	↓22% (200 μM)	–	Ogura et al., 1999 *(399)*
Contractile effect on Ang I, Ang II, Ang III, and Ang IV in endothelium-denuded rat aortic ring preparations pretreated with isoflavones*	↓(10 μM)	No effect (10 μM)	–	Petrescue et al., 2001 *(400)*
Effect on relaxation induced by β1 and β2 adrenoceptor activation (by isoproterenol treatment) in rat aortic rings*	Potentiated the relaxation (1–10 μM)	No effect (10 μM)	–	Satake et al., 2000 *(401)*
Activation of Cl- channel currents in guinea pig ventricular myocytes*	+++ (EC50=~100 μM)	+ (50–500 μM)	–	Shuba et al., 1996 *(402)*
Anaphylactic contraction response to ovalbumin in Isolated bronchial rings from guinea pigs sensitized with purified IgG raised against ovalbumin*	↓(10–100 μM)	No effect (50 μM)	–	Wong et al., 1997 *(403)*
Bronchial contraction induction induced by 0.1 μM leukotriene D4*	↓(~20% control response at 50 μM)	↓(~45% control response at 50 μM)	–	
Endothelin converting enzyme-1 (ECE-1) mRNA expression in OVX rats treated for 4 days with 1 mg/kg injection with genistein or daidzein	↓	↓	–	Rodrigo et al., 2003 *(404)*

Table 78 (continued)

Model	Relative Activity			Reference
	Genistein	Daidzein	Equol or Glycitein	
DNA synthesis in the human endothelial cell line (E304)	↑ (≥0.003 μM)	↑ (≥0.003 μM)	↑(≥0.003 μM equol)	Somjen et al., 2001 *(405)*
Creatinine kinase activity in human vascular smooth muscle cells and/or in the human endothelial cell line (E304)	↑ (≥0.003 μM)	↑ (≥0.003 μM)	↑(≥0.003 μM equol)	
Creatinine kinase activity in the aorta and left ventricle of immature or ovariectomized female rats injected with 500 μg genistein or daidzein	↑	↑	–	
Relaxant response in rabbit basilar artery segments previously contracted with KCl or UTP	EC50=40.7–46.7 μM	EC50=37.1–50.1 μM	–	Torregrosa et al., 2003 *(406)*
Forearm blood flow in healthy human subjects following brachial artery infusion of 10–300 nmol/min genistein or daidzein	↑	No effect	–	Walker et al., 2001 *(407)*
Cell Growth and Genotoxicity				
Induction of structural chromosomal aberrations in cultured human peripheral blood lymphocytes	↑(25 μM)	No effect (100 μM)	–	Kulling et al., 1999 *(408)*
Growth of male human pancreatic tumor cells (HPAF-11)	↑(1 μM)[a]	–	No effect (1–10 μM equol)	Lyn-Cook et al., 1999 *(409)*
Growth of human female pancreatic tumor cells (Su 86.86)	No effect (1 μM)	–	↓ (1 μM equol)	
Cell cycle progression in human gastric cancer cells (HGC-27)*	Arrested at G2/M (60 μM)	Arrested at G1 (200 μM)	–	Matsukawa et al., 1993 *(410)*
Morphological transformation (%) in Syrian hamster embryo cells (SHE)	++++ (12.5–50 μM)	++ (50–200 μM)	–	Tsutsui et al., 2003 *(411)*
Chromosomal aberrations in SHE cells	++ (12.5–50 μM)	− (50–200 μM)	–	
Aneuploidy in SHE cells	++ (12.5–50 μM)	− (50–200 μM)	–	
Somatic mutations in the Na+/K+ ATPase and *hprt* loci in SHE cells	+ (12.5–50 μM)	+ (50–200 μM)	–	
DNA adduct formation in SHE cells	+++ (12.5–50 μM)	+ (50–200 μM)	–	
Cell proliferation in various cancer cell lines of human gastrointestinal origin (HSC-39K6, HSC-40A1, HSC-41E6, HSC-42H, HSC-43C1, HSC-45M2, SH101-P4, HEC-46R1, HCC-48B2, HCC-50D3, ST-Fib, ST-Fib2)	↓(IC50=6.8–25 μg/ml) **[25–93 μM]**	No effect in 10/12 cell lines	–	Yanagihara et al., 1993 *(412)*

Table 78 (continued)

Model	Relative Activity			Reference
	Genistein	Daidzein	Equol or Glycitein	
Growth of the human colon tumor cell line (HCT)	↓(IC50=15 μM)	↓ (IC50=40 μM)	–	Yu et al., 1999 *(413)*
Growth of murine Kl735M2 and human WM451 melanoma cell lines	↓ (30–60 μM)	↓ (30–60 μM)	–	Wang et al., 2002 *(414)*
Cell cycle progression in murine Kl735M2 and human WM451 melanoma cell lines	Cells arrested in G2/M phase (10–60 μM)	• ↑cells in S phase • ↓cells in G1 phase • No effect on G2/M phase (10–60 μM)	–	
Growth of 5 human ovarian cancer cell lines	↓ (9–144 μM)	↓ (9–144 μM)	–	Gercel-Taylor et al., 2004 *(415)*
DNA concentration in benign prostatic epithelial cells primary cultures (% inhibition compared to control)	↓98% (10 μM)	↓80% (10 μM)	↓34% (10 μM equol)	Hedlund et al., 2003 *(416)*
DNA concentration in five human prostate cancer cell lines: 22Rv1, LNCaP, LAPC-4, DU145, and PC-3 (% inhibition compared to control)	No effect–↓51% (10 μM)	10–↓35% (10 μM)	No effect–↓11% (10 μM equol)	
Cell cycle distribution in benign prostatic epithelial cells	Arrested cells in G2/M	Accumulation of cells in G0/G1	Accumulation of cells in G0/G1	
L-type Ca2+ channel current in isolated uterine mooth muscle cells collected from pregnant rats (GD 18–19) Whole-cell voltage clamp*	↓(IC50=50 μM)	No effect (≤300 μM)	–	Kusaka and Sperelakis, 1995 *(417)*
Cell growth in PC-3 prostate tumor cell line (AR-negative)	↓(100 μM)	↓(10–100 μM)	↓(100 μM equol)	Mitchell et al., 2000 *(418)*
Cell growth in LNCaP prostate tumor cell line (AR-positive)	↓(10–100 μM)	↓(100 μM)	↓(10–100 μM equol)	
DNA damage assessed by comet assay in LNCaP prostate tumor cell line (AR-positive) and/or PC-3 prostate tumor cell line (AR-negative)	↑(>10 μM)	No effect (≤500 μM)	↑(>250 μM equol)	
Cell growth in LNCaP prostate tumor cell line (AR-positive)	↓(IC50=40 μM)	↓	–	Onozawa et al., 1998 *(419)*

Table 78 (continued)

Model	Relative Activity			Reference
	Genistein	Daidzein	Equol or Glycitein	
Inflammation/Antioxidant activty				
Mitogen activated protein kinase (MAPK) activity in human platelets*	↑(≥100 μM)	No effect	–	Kansra et al., 1999 *(420)*
Quenching of radicals (number of radicals reduced per molecule determined by electron spin resonance spectroscopy)	"limited"	"limited"	"limited" (equol)	Mitchell et al., 1998 *(421)*
Number of ions reduced per molecule in ferric reducing ability of plasma assay	~0.2	~0.2	~0.4 (equol)	
Antioxidant capacity in Trolox equivalent antioxidant capacity assay, expressed as Trolox equivalents	~1.75	~1.25	~2 (equol)	
Inhibition against ascorbate/ADP/Fe2+ induced lipid peroxidation in vitamin E-deficient liver microsomes	IC50=~900 μM	IC50=~1200 μM	IC50=~700 μM equol	
Ability to protect Caco-2 intestinal cells from hydrogen peroxide and Fe2+-induced lipid peroxidation	No effect (0.1–10 μM)	No effect (0.1–10 μM)	–	Peng and Kuo, 2003 *(422)*
Activity of nitric oxide synthase (iNOS) in lipopolysaccharide-activated RAW 264.7 macrophages	↓(IC50=~50 μM)	↓(IC50=~100 μM)	↓(IC50=~100 μM glycitein)	Sheu et al., 2001 *(423)*
Prostaglandin E2 production in TPA-stimulated rat peritoneal macrophages	↓(IC50=15 μM)	↓(IC50=>30 μM)	↓(IC50=25 μM glycitein)	Yamaki et al., 2002 *(424)*
Immune System				
Immunoglobulin (IgM) levels in mouse splenocyte cultures	↓(≥100 μM)	↑(1000 μM)	–	Han et al., 2002 *(425)*
Immunoglobulin (IgE) levels in mouse splenocyte cultures	↑(≥100 μM)	↑(≥10 μM)	–	
Immunoglobulin (IgA and IgG) levels in mouse splenocyte cultures	No effect (0.01–1000 μM)	No effect (0.01–1000 μM)	–	
Interferon-γ production in mouse splenocytes following stimulation with lipopolysaccharide	↓(≥0.1 μM)	↓(≥0.1 μM)	–	Nakaya et al., 2003 *(426)*

Table 78 (continued)

Model	Relative Activity			Reference
	Genistein	Daidzein	Equol or Glycitein	
Neurocellular and Neuroendocrine System				
Astrocyte stellation induction*	↑(≥10 μM)	No effect (1–100 μM)	–	Abe and Saito, 1999 *(427)*
Dopamine release from mouse striatum*	↑(≥100 μM)	No effect (200 μM)	–	Bare et al., 1995 *(428)*
Expression of GABAA receptors on the membrane of *Xenopus* ooctyes injected with rat brain mRNA*	↓(200 μM)	No effect (200 μM)	–	Balduzzi et al., 2001 *(429)*
Effect on the up-regulation of α1B-adrenoceptor expression induced by epinephrine and staurosporine in clone H99 of transfected Chinese hamster ovary cells*	↓(≥11 μM)	↓(≥118 μM)	–	Bird et al., 1997 *(430)*
GABA-evoked current in frog melanotrophs regulated by GABAA receptor*	↑at 0.01–10 μM; ↓at 100 μM	No effect	–	Castel et al., 2000 *(431)*
Function of recombinant mouse GABAA receptors expressed in *Xenopus* oocytes or HEK cells assessed by voltage or patch clamp*	↓(100 μM)	↓(100 μM)	–	Dunne et al., 1998 *(432)*
Glycine-activated current in rat hypothalamic neurons*	↓(≥50 μM)	↓(≥100 μM)	–	Huang and Dillon, 2000 *(433)*
GABA-activated currents from human embryonic kidney culture (HEK293 cells) expressing rat α1β2γ2S or α1β2 receptors*	↓(100 μM)	↓(≥100 μM)	–	Huang et al., 1999 *(434)*
Whole-cell patch-clamp recordings in rat diagonal band of Broca neurons*	Attenuated response to GABAA agonist, muscimol (100 μM)	No effect on response to GABAA agonist, muscimol (100 μM)	–	Jassar et al., 1997 *(435)*
Polypeptide levels of *N*-methyl-D-aspartate receptor (NMDA) NR2B subunit in cortical neurons collected from mice*	↓(100 μM)	No effect (100 μM)	–	Kalluri and Ticku, 1999 *(436)*
Neurotoxin-induced 22N+ influx through voltage-sensitive Na+ channels in cultured rat brain neurons*	↓(IC50=60 μM)	↓(IC50=195 μM)	–	Paillart et al., 1997 *(437)*
Voltage-dependent K+ channel gating in cultured mouse Schwann cells (slowly inactivating delayed-rectifier K+ current and transient K+ current)	↓(100 μM)	No effect (100 μM)	–	Peretz et al., 1999 *(438)*

Table 78 (continued)

Model	Relative Activity			Reference
	Genistein	Daidzein	Equol or Glycitein	
Other				
PPARγ-dependent gene transcription in human umbilical vein endothelial cells (HUVEC)	↑(1 μM)	↑(1 μM)	↑(1 μM equol)	Chacko et al., 2007 *(448)*
Inhibition of glucose efflux in human erythrocytes at pH of 6.5	*Ki*=3.92 μM	*Ki*=18.62 μM	–	Martin et al., 2003 *(449)*
ACTH-stimulated cortisol production by primary cultures of human fetal and postnatal adrenal cortical cells	↓(0.4–40 μM)	↓(0.4–40 μM)	–	Mesiano et al., 1999 *(450)*
ACTH-stimulated DHEA-S production by primary cultures of human fetal adrenal cortical cells	No effect	No effect	–	
ACTH-stimulated DHEA and DHEA-S production by primary cultures of human postnatal adrenal cortical cells	↑(1–4 μM)	↑(1–4 μM)	–	
Inhibition of recombinant SULT1A1 from rat liver	IC50=1.1 μM	IC50=4.6 μM	–	Mesia-Vela and Kauffman, 2003 *(451)*
Inhibition of recombinant SULT1A1 from rat liver	IC50=106 μM	IC50=116 μM	–	
Inhibition of cytosolic sulfotransferase from rat liver	IC50=0.8 μM	IC50=2.2 μM	–	
Inhibition of E2–3β- glucuronide by rat liver microsomal UGTs	IC50=51 μM	IC50=188 μM	–	
Inhibition of E2–17β- glucuronide by rat liver microsomal UGTs	IC50=261 μM	IC50=236 μM	–	
Insulin release in INS-1 cells (short-term effect)*	↑(100 μM)	No effect (100 μM)	–	Neye and Verspohl, 1998 *(452)*
Cortisol secretion from adrenocortical H295R cells induced by dibutyryl cAMP	↓(≥9.6 μM)	↓(≥10.2 μM)	–	Ohno et al., 2002 *(453)*
Glucose uptake in myelocytic U937 cells	↓(≥5 μM)	↓(≥10 μM)	–	Park, 1999 *(454)*
Glucose-stimulated insulin secretion in adult rat islet cells*	↓(at 100 μM)	No effect (at 100 μM)	–	Persaud et al., 1999 *(455)*
PPARγ-dependent gene transcription based on luciferase activity in RAW 264.7 cells containing a PPARγ expression plasmid	↑(9.3 μM)	↑(9.8 μM)	–	Mezei et al., 2003 *(456)*
Insulin release in the insulin secreting cell line (INS-1) in the presence of glucose*	↑(EC50=~20 μM)	No effect	–	Verspohl et al., 1995 *(457)*

*Study assessed impact of tyrosine kinase inhibition on pathway

Table 79. In Vitro Genetic Toxicity Studies of Genistein

Concentrations Tested	*Metabolic Activation*	*Species or Cell Type/Strain*	*Endpoint*	*Results*	*Reference*
≤ 100 µg **[0.37 µmol/plate]**	Yes	*Salmonella typhimurium* strains TA1538, TA98, TA100	Mutation	↔ With and without metabolic activation	Reviewed in Munro et al., 2003 *(178)*
Genistein 19.5–1250 µg **[0.072–4.6 µmol]** /plate in bacteria and 0.3–300 µg/mL **[1.1–1110 µM]** in lymphoma cells Administered as a purified isoflavone product containing 40–50% genistein, 18–25% daidzein, and 1–4% glycitein	Yes	*Salmonella typhimurium* strains TA 1535, TA1537, TA98 and TA100 *E. coli* WP2uvrA	Mutation	Weak ↑ at 39.1–312.5 µg/plate in TA100 with metabolic activation ↔ in other strains and without metabolic activation	Misra et al., 2002 *(463)*
		Mouse lymphoma cells	Mutation	↑ at ≥1.2 µg/mL in lymphoma cells with activation ↑ at ≥12 µg/mL in lymphoma cells without activation	
10–3333 µg/plate **[0.12–12.3 µmol/plate]**	Yes	*Salmonella typhimurium* strains TA1535, TA97, TA98, TA100, TA102	Mutation	↔	McClain et al., 2006 *(464)*
0.813–60 µg/mL **[0.003–0.22 µmol/mL]**	Yes	Mouse lymphoma cells	Mutation	↑ at ≥0.813 µg/mL with activation ↑ at ≥3.250 µg/mL without activation	
10–80 µM **[2700–21,619 µg/L]**	No	L5178Y mouse lymphoma cells	Mutation	↑ at 10–80 µM	Boos and Stopper, 2000 *(465)*
10–25 µM **[2700–6760 µg/L]**	No	Chinese hamster V79 cells	Mutation	Marginal ↑ at 25 µM	Kulling and Metzler, 1997 *(466)*
1–20 µg/ml	No	Human lymphoblastoid cell lines (AHH-1 tk+/– and L3)	Mutation	↑ at 5–20 µg/ml in AHH-1 tk+/– cells but not L3 cells	Morris et al., 1998 *(467)*
5–75 µM **[1350–20,270 µg/L]**	No	Chinese Hamster V79 cells	Micronuclei	↑ at 5–25 µM and ↓ at ≥50 µM ↓ most likely due to cytotoxicity	DiVirgilio et al., 2004 *(468)*
5–25 µM **[1350–6760 µg/L]**	No	Chinese hamster V79 cells	Micronuclei	**[↑ at ≥5 µM]**	Kulling and Metzler, 1997 *(466)*
50 µM	No	Chinese Hamster V79 cells	Micronuclei	↑ at 50 µM	Snyder and Gillies, 2002 *(469)*
12.5–100 µM **[3380–27,020 µg/L]**	No	L5178Y mouse lymphoma cells	Micronuclei	↑ 12.5–100 µM	Boos and Stopper, 2000 *(465)*

Table 79 (continued)

Concentrations Tested	*Metabolic Activation*	*Species or Cell Type/Strain*	*Endpoint*	*Results*	*Reference*
1–20 µg/ml	No	Human lymphoblastoid cell lines (AHH-1 tk+/– and L3)	Micronuclei	↑ at 10–20 µg/ml in both cell lines	Morris et al. 1998 *(467)*
25 µM **[6760 µg/L]**	No	Human lymphocytes	Chromatid breaks, gaps and interchanges	↑	Kulling et al., 1999 *(408)*
10–100 µM	No	Human lymphocytes	Chromosomal aberrations	↑ at 50 and 100 µM	Abe, 1999 *(470)*
50 µM **[13,520 µg/L]**	No	MLL gene from human hemato-poietic cells	Gene cleavage	↑	Strick et al., 2000 *(471)*
1–200 µM **[270–54,050 µg/L]**	No	Human sperm and lymphocytes	DNA strand breaks	Variable results with some ↑ in lymphocytes at ≥50 µM ↓ in sperm	Anderson et al., 1997 *(472)*
10–500 µM **[2700–135,120 µg/L]**	No	LNCaP and PC-3 human pros-tate tumor cells	DNA strand breaks	↑ at < 10–100 µM in LNCaP cells and < 10–250 µM in PC-3 cells	Mitchell et al., 2000 *(418)*
100–500 µM **[27,000–135,120 µg/L]**	No	V79 cells	DNA strand breaks	↑ ≥250 µM 50 µM did not ↓ strand breaks induced by hydrogen peroxide	DiVirgilio et al., 2004 *(468)*
7–118 µM **[1890–31,890 µg/L]**	No	L5178Y mouse lymphoma cells	DNA strand breaks	↑ at 7–118 µM	Boos and Stopper, 2000 *(465)*
100 µM	No	HT29 human colon tumor cells	DNA strand breaks	↑ at 100 µM	Pool-Zobel et al., 2000 *(473)*
10–50 µM	No	Human lymphocytes	DNA strand breaks	↑ at 10–50 µM	Ullah et al., 2009 *(459)*

↑, ↓, ↔ = Statistically significant increase, decrease, or no significant effect.

Table 80. Results of In Vivo Genetic Toxicity Studies of Genistein

Species	Dose (Route)	Cell Type	Endpoint	Results	Reference
Swiss-Webster mouse	500–2000 mg/kg bw genistein by gavage Administered as a purified isoflavone supplement that also contained daidzein and glycitein	Bone marrow erythrocytes	Micronuclei	Small, non-dose dependent ↑ in males but not females Similar findings reported for historical controls.	Misra et al., 2002 *(463)*
Swiss albino mouse	Mice were administered a single oral dose of 40 mg/kg bw isoflavones obtained from a supplement containing 33 mg genistein and 67 mg daidzein/100 mg product. **[Based on percentages of each individual isoflavone, the genistein dose was estimated at 13.2 mg/kg bw.]**	Bone marrow	Chromosomal aberrations and micronuclei	↔	Khan et al., 2005 *(474)*
C57BL6J mouse	20 mg/kg/day for 5 days (oral)	Splenocytes	Micronuclei	↔	Reviewed in Munro et al., 2003 *(178)*
ICR female mouse	50 mg/kg bw IP at 12 hour intervals for 3 days	Bone Marrow	Sister chromatid exchanges	↑	Giri and Wu, 1995 *(475)*
MORO mouse	0.2–20 mg/kg/day by gavage for 14 days	Blood	Micronuclei	↔	McClain et al., 2006 *(464)*
RAIF rat	500–2000 mg/kg bw by gavage	Bone marrow	Micronuclei	↔	
Wistar rat	2000 mg/kg bw by gavage	Bone marrow	Micronuclei	↔	
Sprague Dawley rat	250 ppm in diet from PND 21–35	Mammary cells	Micronuclei, hyperdiploidy, and polyploidy	↔	Uppala et al., 2005*(476)*

↑, ↓, ↔ = Statistically significant increase, decrease, or no significant effect.

Using the comet assay, one study reported evidence of *in vitro* genetic toxicity in daidzein-treated human sperm and lymphocytes. Bacterial tests for gene mutations (Ames assay) were negative for daidzein. One daidzein study in mice, using i.p. injection of 50 mg/kg bw/day for 3 days reported an increase in sister chromatid exchanges in bone marrow cells. Equol and 3 other metabolites of daidzein were shown to induce micronuclei in L5178Y cells. Glycitein was negative in an *in vitro* micronucleus test **[assumed to be in L5178Y cells based on other work from this laboratory]**.

2.4 Carcinogenicity

2.4.1 NTP Rodent Bioassay

The NTP *(477)*, supported by an interagency agreement between the FDA and the NIEHS, examined the carcinogenic effects of genistein on Sprague Dawley Rats in a 2-year carcinogenesis study. The study was conducted in compliance with the FDA GLPs. At weaning, subsets of Sprague Dawley/CD23/NCTR BR rats from the F_1 and F_3 generations of the multigeneration study were assigned to the 2-year study. The dose levels for the multigeneration study were based on the results of prior short-term studies; the high dose was selected to avoid significant maternal toxicity but still induce mild reproductive tract lesions in the offspring and the low dose was not expected to produce significant effects. Study animals received diets containing 0 (control), 5, 100, or 500 ppm genistein (purity > 99%). The base diet was an alfalfa- and soy-free rodent feed (5K96, Purina Mills, Inc, Richmond, IN) containing casein in place of the soy and alfalfa protein. The levels of genistein and daidzein in the control diet were ≤0.6 ppm each, consistent with the isoflavone intake of humans consuming typical Western diets. Bedding consisted of heat-treated hardwood chips. Three exposure regimens were studied (n=50/sex/group/exposure regimen): continuous exposure from conception through 2 years (F_1C–F_1 generation–756 days of exposure), exposure from conception through PND 140 followed by control diet until termination (F_1T140–F_1 generation–161 days of exposure), and exposure from conception through weaning at PND 21 followed by control diet until termination (F_3T21–F_3 generation–42 days of exposure); the F_1C and the F_1T140 regimens shared a common control. During pregnancy, the ingested genistein doses of the dams were approximately 0.5, 9, or 45 mg/kg/day; during lactation, the dams' ingested doses were 0.7, 15, or 75 mg/kg/day (supplemental studies have indicated minimal transfer of genistein through mother's milk); the mean directly ingested genistein doses during the period prior to PND 140 (F_1C and F_1T140 regimens only) were approximately 0.4, 8, or 44 mg/kg/day for females and 0.4, 7, or 37 mg/kg/day for males. For the period between PND 140 and the end of the study (F_1C regimen only), mean ingested doses were approximately 0.3, 5, or 29 mg/kg/day for females, and 0.2, 4, or 20 mg/kg/day for males. Clinical observations, body weights and feed consumption were monitored throughout the study. Beginning at 5 months of age, one-half of the females in each exposure group were subjected to vaginal smears for 5 consecutive days once per month until the female exhibited aberrant estrous cycling. All surviving animals were killed after two years and complete necropsies were performed. At terminal sacrifice, routine organs were weighed, and selected tissues were fixed and processed for histopathological examination. Survival and estrous cycle data were analyzed within each of the three exposure regimens using a log-rank test for homogeneity and Tarone's test for overall trend. A mixed models approach to repeated measures ANOVA was used to analyze body weights and feed consumption. Terminal body weights were also used as a covariate in an ANOVA procedure for organ weight analyses; for each endpoint analyzed, Dunnett's two-sided test was used to compare the control group mean to each exposed group mean. Analyses of the incidences of neoplastic lesions were conduvcted separately for each exposure regimen: the Poly-k test was used to assess neoplasm

prevalence, tests of significance included pairwise comparisons of each exposed group with controls and tests for overall exposure concentration-related trends. With the exception of the incidences of hyperplasia in the male mammary gland, nonneoplastic lesions were not statistically evaluated, the hyperplasia data were analyzed by a Jonckheere-Terpstra test for increasing trend.

Survival, Body Weights, Feed Consumption and Clinical Observations: There were no consistent effects of genistein exposure on survival. Mean body weights of the 500 ppm F_1C (females only) and the 500 ppm F_1T140 rats were less than the controls; mean body weights of the F_3T21 rats were generally similar to the controls, throughout the study. Other than a minimal depression of feed consumption during several early weeks in F_1C and F_1T140 females, genistein did not show any consistent effects on feed consumption in this study. There were no exposure-related clinical observations.

Estrous: For all exposure regimens, exposure to 500 ppm genistein resulted in an acceleration of the onset of aberrant cycles that was highly significant. In addition, the F_3T21 animals showed a significant effect of exposure to 5 ppm. In all cases, the prevalent stage that caused the judgment of aberrant cycling was estrus. *Organ Weights*: Significant effects of genistein exposure on organ weights in males were limited to the prostate gland and the liver. Prostate and liver weights were significantly higher in the 500 ppm F_1C males than their controls; however, liver weights of the 100 ppm and the 500 ppm F_3T21 males were significantly lower. In female rats, the weights of the pituitary gland were significantly higher in the 500 ppm F_1C, 500 ppm F_1T140, and the 100 ppm F_3T21 groups, than their respective controls. *Histopathology*: For the most part, the nonneoplastic lesions observed were consistent with lesions typically observed in aging Sprague-Dawley rats. (see **Table 81** for incidences of selected neoplasms and nonneoplastic lesions. In F_1C females, there was a significant positive trend in the incidence of mammary gland adenoma or adenocarcinoma (combined), and the incidence in the 500 ppm group was significantly greater than that in the control group. Incidences of mammary gland fibroadenoma in F_1C females occurred with negative trends, and the incidence in the 500 ppm group was significantly less than that in the controls. The incidence of fibroadenoma was marginally less in 5 ppm F_3T21 females than in the controls. No incidences of fibroma or fibroadenoma occurred in control males in any of the exposure regimens. Two 500 ppm F_3T21 males were diagnosed with fibroma, and two were diagnosed with fibroadenoma; there was no significant treatment effect. Positive trends in the incidences of mammary gland hyperplasia were observed in the F_1C and the F_1T140 males; a separate publication from this study details the effects of exposure on male mammary hyperplasia *(478)* and is summarized elsewhere in the report.

Pituitary Gland (Pars Distalis): In F_1T140 males, a significant negative trend occurred in the incidences of adenoma; the incidence in the 100 ppm group was significantly less than the controls. The incidence of adenoma was significantly lower in the 5 ppm F_1C males. Significant positive trends occurred in the incidences of adenoma or carcinoma in F_1C and F_1T140 females, and the incidence was significantly higher in the 500 ppm F_1C group. See **Table 82** for a summary of the incidence of adenoma in the pituitary gland.

Pancreatic islets: A significant positive trend occurred in the incidences of adenoma or carcinoma in F_1C males, but no group was significantly different than the controls; there is little evidence that the slightly higher incidences of these lesions are biologically meaningful. *Preputial Gland:* A significant positive trend occurred in the incidences of squamous cell carcinoma in F_3T21 males, and the incidence in the 100 ppm group was significantly greater than the controls; the incidences were not considered related to genistein exposure.

Table 81. Incidences of Selected Neoplasms and Nonneoplastic Lesions of the Mammary Gland in Rats in the Two-year Feed Study of Genistein (NTP Multigenerational Cancer Bioassay)

Exposure Level, ppm	Male				Female			
	0	5	100	500	0	5	100	500
F_1C[a]								
Fibroadenoma[b]	0.0% (n=41)	2.3% (n=43)	0.0% (n=40)	4.8% (n=42)	59.3% (n=54)	54.0% (n=50)	56.0% (n=50)	24.5%** (n=49)
Adenoma or Adenocarcinoma[b]	–	–	–	–	16.7%	8.0%	16.0%	32.7%*
F_1T140[a]								
Fibroadenoma[b]	0.0% (n=41)	0.0% (n=42)	0.0% (n=34)	0.0% (n=45)	59.3% (n=54)	60.0% (n=50)	62.0% (n=50)	50.0% (n=50)
Adenoma or Adenocarcinoma[b]	–	–	–	–	16.7%	6.0%	10.0%	20.0%
F_3T21								
Fibroma	0.0% (n=39)	0.0% (n=43)	0.0% (n=41)	4.9% (n=41)	–	–	–	–
Fibroadenoma[b]	0.0%	0.0%	0.0%	4.9%	60.4% (n=53)	40.8%* (n=49)	66.0% (n=50)	56.0% (n=50)
Adenoma or Adenocarcinoma	–	–	–	–	13.2%	16.3%	22.0%	26.0%

[a]A single group of animals served as a common control for the F_1C and the F_1T140 regimens.
[b]Significant Trend–Females
* Statistically significant $P < 0.05$
** Statistically significant $P < 0.001$
– No value presented

Table 82. Incidences of Adenoma of the Pituitary Gland (Pars Distalis) in Rats in the Two-year Feed Study of Genistein (NTP Multigenerational Cancer Bioassay)

Exposure Level, ppm	Male				Female			
	0	5	100	500	0	5	100	500
F_1C [a]								
Adenoma [b]	46.9% (n=49)	30.4%* (n=46)	44.0% (n=50)	44.9% (n=49)	70.3% (n=54)	80.0% (n=50)	66.0% (n=50)	93.9%** (n=49)
F_1T140 [a]								
Adenoma [b, c]	46.9% (n=49)	36.7% (n=49)	29.1%* (n=48)	33.3% (n=48)	70.3% (n=54)	65.3% (n=49)	80.0% (n=50)	86.0% (n=50)
F_3T21								
Adenoma	34.7% (n=49)	39.1% (n=46)	35.4% (n=48)	31.3% (n=48)	77.4% (n=53)	84.0% (n=50)	84.0% (n=50)	80.0% (n=50)

[a] A single group of animals served as a common control for the F_1C and the F_1T140 regimens.
[b] Significant Trend – Females
[c] Significant Trend – Males
* Statistically significant $P < 0.05$
** Statistically significant $P < 0.001$

Adrenal medulla: Significant positive trends occurred in the incidences of benign pheochromocytoma in F_1C males, and significant positive trends occurred in the incidences of benign, complex or malignant pheochromocytoma in F_1C and F_1T140 males. The incidence of benign pheochromocytoma was slightly higher (not statistically significant) in 500 ppm F_1C males, the incidences of complex or malignant pheochromocytoma were higher in 500 ppm F_1C and F_1T140 males compared to the controls. Lower incidences of benign or complex pheochromocytoma occurred in females, and no statistically significant exposure effects were seen in females. There is little evidence that genistein exposure affects the incidences of adrenal medulla pheochromocytomas. *Nose:* A significant positive trend occurred in the incidences of squamous cell carcinoma in F_1C males. Low incidences of this neoplasm occurred sporadically in exposed groups of males and in control and exposed groups of females; available evidence suggests that incidences of squamous cell carcinoma of the nose were not related to genistein exposure.

The NTP concluded there was equivocal evidence of carcinogenic activity of genistein in female Sprague-Dawley rats based on marginally increased incidences of pituitary gland neoplasms. Under the conditions of this 2-year feed study where offspring of three prior generations of animals exposed to the test compound were exposed from conception through weaning (PND 21) followed by control feed until termination (F_3T21), there was no evidence of carcinogenic activity of genistein in male Sprague-Dawley rats exposed to 5, 100, or 500 ppm. There was equivocal evidence of carcinogenic activity of genistein in female Sprague-Dawley rats based on increased incidences of mammary gland adenoma or adenocarcinoma (combined). Exposure to genistein was also shown to accelerate the onset of aberrant estrous cycles in female Sprague-Dawley rats whether exposures were continuous or truncated at PND 140 or at weaning. The effects of genistein on estrous cycling and the incidences of common hormonally related spontaneous neoplasms of female Sprague-Dawley rats are consistent with an estrogenic mechanism of toxicity.

2.4.2 Breast cancer

Epidemiology

Breast cancer rates are lower in Asian than Western populations. Adjusted incidence rates per 100,000 women range from 28.7 in New Delhi, 27.2 in Shanghia, 36.2 in Hong Kong, 36.3 in Hiroshima, 20.8 in Seoul to 68.9 in Austria, 78.5 in Canada, and 103.9 in the U.S. (Parkin 2003 as reviewed in Tomar and Shiao, 2008 *(479)*). Breast cancer rates are higher in Asian women who immigrate to the US prior to age 35 and in Asian women born in the US than in women born in Asian countries who do not immigrate to the US. This observation is often interpreted as reflecting a primary influence for environmental or dietary effects, with any possible genetic components contributing a much weaker effect. In this context, higher soy product intake in Asian women has been implicated as one possible reason for the lower breast cancer rates in Asian populations *(284; 480; 481)*, although Tomar and Shiao, 2008 *(479)* note that breast cancer incidence in Asian women of Indian descent is similar to other Asian populations and there was little or no soy consumption in India until the 1990s. A number of factors aside from differential patterns of soy consumption could contribute to international differences in rates of breast cancer, such as age at menarche, parity, age at first birth, breastfeeding, genetics, and lifestyle *(479)*. Nonetheless, each of these factors are generally weak individual predictors of breast cancer risk, and many breast cancers arise in women who exhibit none of these established risk factors.

Genistein has been widely reported to activate ERα and act as an estrogen and mitogen in experimental breast cancer models. While estrogens have been widely implicated in affecting breast cancer risk, the data on estrogen exposure and breast cancer risk is complicated. There is general consensus that the association of increased breast cancer risk with early onset of menarche, late onset of menopause, nulliparity, or post-menopausal obesity reflect greater lifetime exposures to endogenous estrogen (reviewed in *(482)*). These observations are also broadly consistent with the evidence that current or recent use of an estrogen-based hormone replacement therapy also increases breast cancer risk *(483)*. However, estrogen-alone therapy, usually administered in the form of conjugated equine estrogens (CEE) is less potent in this regard than a combined therapy that includes an estrogen and a progestogen *(483)*. In women with a prior hysterectomy (41% of whom had prior bilateral ovariectomy), combined estrogen-progestogen formulations, but not estrogen-alone hormone replacement therapy, significantly increased breast cancer risk in postmenopausal women *(484)*. Of note, the estimated increases in breast cancer risk are associated only with current or recent use, and the risk declines to baseline over time among prior users.

A relatively large number of epidemiological studies have been conducted to determine if isoflavone or soy intake is related to breast cancer incidence. Reviews of these studies report conflicting results, with some concluding the studies provide evidence of protective effects of soy product intake and others concluding no overall or robust effect. While simple literature reviews can be useful, appropriately conducted statistical meta-analyses generally provide the most useful insights into associations between isoflavone or soy consumption and breast cancer risk. Meta analysis is a quantitative procedure that combines the data across multiple individual studies usually with two primary goals in mind. Firstly, these analyses use the increased statistical power of greater numbers to derive potentially more robust summary estimates of associations within the data, often the most widely cited use of these techniques. Secondly, meta-analysis attempts to identify factors that may explain apparent inconsistencies or confounders in the outcomes of individual studies. The identification of these factors can provide guidance for improvements in the design of subsequent studies. Two rigorous statistical meta analyses

have been conducted to date exploring the associations among soy intake and breast cancer risk; Trock *et al.*, 2006, *(485)* and Wu *et al.*, 2008 *(486)*. We found no meta analyses studying the association of soy intake and the risk of breast cancer mortality; lack of clarity on the role of hormone replacement therapies in affecting breast cancer mortality also is evident *(483)*.

Trock *et al.*, 2006 *(485)*, supported by the National Cancer Institute and the American Institute for Cancer Research, performed a meta-analysis of 18 epidemiological studies (12 case-control and 6 cohort or nested case-control designs) that examined the relationship between soy intake and breast cancer. Risk estimates, measures of soy exposure, and control for confounding factors varied across the studies. Among all women, high soy intake was modestly associated with reduced breast cancer risk (OR=0.86, 95% CI=0.75-0.99). When considered independently, there was no significant association in studies of women in Asian countries (OR=0.89, 95% CI=0.71-1.12). Because exposure to soy isoflavones is very different in Western and Asian cultures, the finding of a similar magnitude of effect in both populations led the authors to suggest artifact, differential levels of confounding and misclassification, or true differences based on timing of exposure. The inverse relationship between soy intake and breast cancer was stronger in premenopausal women (OR=0.70, 95% CI=0.58-0.85) than in postmenopausal women (OR=0.77, 95% CI=0.60-0.98). When soy protein intake in grams per day was used to assess exposure, the only statistically significant association was observed in premenopausal women (OR=0.94, 95% CI=0.92-0.97). The authors concluded that soy intake may be associated with a small reduction in breast cancer risk; however, the results should be viewed with caution given the potential for exposure misclassification, confounding, and lack of dose response.

Wu *et al.*, 2008 *(486)*, supported by the California Breast Cancer Research Program and the Susan G Koman Breast Cancer Foundation, published a meta-analyses of epidemiological studies of soy and breast cancer that was limited to studies that had a relatively complete dietary assessment of soy exposure and appropriate statistical consideration of potential confounders. **[Although there was some overlap, the studies reviewed by Wu et al., 2008 *(486)* were not the same studies as those reviewed by Trock et al., 2006 *(485)*[10].]** Two analyses were conducted, one on 8 studies (1 cohort, 7 case-control) in Asian populations with high soy consumption and the other on 11 studies (4 cohort or nested case-control, 7 case-control) in Western populations with low levels of soy consumption[11]. In contrast to the analysis by Trock *et al.*, 2006, analysis of the Asian studies showed a significant trend of decreasing risk with increasing soy food intake. For example, a 29% risk reduction was found among women with high estimated intake (≥20 mg isoflavones per day) compared to the lowest level of soy food intake of ≤5 mg isoflavones per day (OR=0.71, 95% CI=0.60-0.85). This represented ~16% risk reduction per 10 mg of isoflavones consumed per day. Modest intake of 10 mg per day

[10] The 18 epidemiological studies included in the meta-analysis performed by Trock *et al.*, 2006 [485] were: Lee (1992), Hirose (1995), Yuan (1995), Wu (1996), Greenstein (1996), Ingram (1997), Witte (1997), Chie (1997), Key (1999), Dai (2001), Shu (2001), Horn-Ross (2001), den Tonkelaar (2001), Wu (2002), Horn-Ross (2002), Yamanoto (2003), Linseisen (2004), and Grace (2004).

[11] A total of 19 studies were considered in the meta-analysis conducted by Wu *et al.*, 2008 [486]. The 8 studies conducted in Asian populations were: Lee (1991), Dai (2001), Wu (2002), Yamamoto (2003), Hirose (2005), Lee (2005), Shannon (2005), and Do (2007). The 11 studies conducted in Western populations were: Witte (1997), Horn-Ross (2001), Horn-Ross (2002), Linseisen (2003), Peterson (2003), Dos Santos Silva (2004), Grace (2004), Keinan-Boker (2004), Bosetti (2005), Touilaud (2005), and Fink (2007)

was associated with a 12% decrease in breast cancer risk. Two of the studies in Asian studies, Shu *et al.*, 2001 *(487)* and Wu *et al.*, 2002 *(488)*, reported a greater risk reduction with soy intake during adolescence compared to adult intake. Soy intake was not related to breast cancer risk in the studies of Western populations where the range of average low and high isoflavone intakes were 0.15 and 0.8 mg per day (OR=1.04, 95% CI=0.97-1.11).

While the Trock *et al.*, 2006 and Wu *et al.*, 2008 meta analyses combined similar but not identical studies, both suggest a modest but potentially protective effect of soy consumption for some women with respect to breast cancer risk. However, they appear to differ with respect to which women are most likely to receive benefit, perhaps reflecting the acknowledged inconsistent and heterogeneous nature of the studies they combined [479-481; 485]. For example, Wu *et al.*, 2008 *(486)* did not detect indications of a modifying effect by menopausal status whereas others have reviewed the literature and found some evidence, based mostly on case-control studies, of a protective role in premenopausal women compared to postmenopausal women *(481; 485)*. A modifying effect of menopausal status is consistent with one hypothesis that phytoestrogens may have stimulatory effects in low-estrogen states such as menopause, but inhibitory effects on estrogen in high-estrogen states such as the premenopause. De Lemos reviewed the literature on phytoestrogens on breast cancer growth and concluded that low concentrations of genistein and daidzein were generally stimulatory *in vitro* and *in vivo* animal studies and antagonized the effect of tamoxifen *in vitro*. At high concentrations genistein may inhibit tumor cell growth and could enhance the effect of tamoxifen *in vitro* but these concentrations may not be achievable *in vivo (489)*.

Several non-statistical reviews of the literature have also explored the potential association between soy consumption and breast cancer risk. Messina and Wood reviewed 11 clinical studies of isoflavones and soy protein that included measures of breast cancer risk obtained from breast biopsies, mammograms, or nipple aspirate fluid *(490)*. [**A competing interest is noted in the paper. The first author is president of Nutrition Matters, Inc., a "nutrition consulting company with clients involved in the manufacture and/or sale of soyfoods and isoflavone supplements".]** Their overall conclusion from these studies was that *"there is little clinical evidence to suggest that isoflavones will increase breast cancer risk in healthy women or worsen the prognosis of breast cancer patients."* Further, they concluded there was *"no evidence that isoflavone intake increases breast tissue density in pre- or postmenopausal women or increases breast cell proliferation in postmenopausal women with or without a history of breast cancer."* While they discussed the finding reported by Petrakis *et al.*, 1996 of hyperplastic epithelial cells in the nipple aspirate fluid in pre- and post-menopausal women following treatment with isolated soy protein with isoflavones, Messina and Wood noted that the Petrakis 1996 was a pilot study with significant drop-out rates. However, it is not clear that these events would affect the primary observations of the changes within the mammary gland. Messina and Wood further noted that the finding of hyperplastic epithelial cells persisted long after discontinuation of soy treatment. Messina and Wood concluded that the broader epidemiological literature on soy or isoflavone intake also did not provide an indication of increased risk.

Balk *et al.*, 2005 *(80)* reviewed 13 clinical trials designed to evaluate whether soy protein and/or isoflavone intervention alters risk factors for the development of breast cancer. Participants recruited for these studies did not have a diagnosis of cancer and the development of cancer was not reported as an outcome. Outcomes assessed in these studies were typically related to effects on estrogens and other reproductive

hormones, estrogen metabolites, or other indicators of estrogenicity such as vaginal cell maturation, estrogen receptor expression, epithelial cell proliferation in the breast, and mammographic breast density. Balk *et al.*, 2005 concluded that no causal relationship could be established between these markers and cancer because they are not established risk factors for the disease. Three of the clinical trials reviewed by Balk *et al.*, 2005 raised concern that isoflavones may exert estrogenic effects on breast tissue. The specific findings were (1) an increase in the presence of epithelial hyperplasia in nipple aspirate fluid from pre- and post-menopausal women following a 6-month soy protein intervention (Petrakis 1996 as reviewed in Balk *et al.*, 2005 *(80)*), and (2) changes in estrogen-regulated proteins in nipple aspirate fluid that were consistent with an estrogenic response among pre-menopausal women with benign or malignant breast disease following a 14-day soy protein intervention (Hargreaves 1999 and McMichael-Phillips 1998 as reviewed in Balk *et al.*, 2005 *(80)*). Stimulation of cell proliferation could be predicted to increase the density of breast tissue; however, neither of the two trials that assed mammographic density reported any effect of soy intervention (Maskarinec 2003 and 2004 as reviewed in Balk *et al.*, 2005 *(80)*). The National Cancer Institute is currently funding two randomized double-blind studies of soy isoflavones that will include measurements of breast density in premenopausal women taking soy isoflavones for 2-years (NCT00204490) and breast epithelial cell proliferation in women considered high risk for breast cancer taking genistein for 6 months (NCT00290758) (reviewed in *(481)*).

In the 2007 World Cancer Research Fund/American Institute for Cancer Research sponsored "Food, Nutrition, Physical Activity, and the Prevention of Cancer: a Global Perspective" *(491)* the reviewers determined that for both premenopausal and postmenopausal breast cancers the data were either too inconsistent, too sparse, or of too poor quality to allow a conclusion to be reached regarding soy product consumption and breast cancer risk.

The American Heart Association Nutrition Committee *(252)* also considered the issue of cancer in its evaluation and concluded that the efficacy and safety of soy isoflavones in the prevention or treatment of breast and uterine endometrial has not been established. With respect to breast cancer, the Committee concurred with the conclusions of others that research remains insufficient to know whether certain phytoestrogens are protective or harmful for breast cancer and at what dose and life-stage they may be active. The evidence from clinical trials was considered "meager" and provided some cautionary data with respect to possible adverse effects.

The American Cancer Society (ACS) provides plain-language guidance on treatment options for cancer patients. With respect to soy isoflavones and estrogen receptor-positive breast cancer, the ACS considers the research unclear on whether soy isoflavones may act as anti-estrogens and reduce cancer growth or whether their weak estrogenic activity could cause cancers to grow faster. The ACS notes that many oncologists recommend that people who are taking tamoxifen or aromatase inhibitors, or people with estrogen-sensitive breast tumors should avoid adding large amounts of soy, including soy supplements or isoflavones, to their diets. *http://www.cancer.org/docroot/ETO/content/ETO_5_3x_Soybean.asp*.

While evidence from some individual studies can be read as implying a potential risk for the development of breast lesions, results from both of the published meta-analyses show little evidence that the risk of developing breast cancer is increased among either Asian or Caucasian populations. Evidence for a reduction in breast cancer incidence associated with the consumption of soy products can also be extracted from individual studies. While both meta-analyses found evidence in support of a potential reduction in risk, they

were not consistent with respect to the populations that might receive benefit. While the weight of evidence remains more suggestive or a potentially protective effect in some women, no compelling conclusions for either increased or reduced breast cancer risk can yet be drawn from the existing body of literature. There is no data to allow for an assessment of the effects of soy exposure on breast cancer mortality.

Animal and in vitro studies

There are several types of animal models available for breast cancer researchers that include xenografts of human breast cancer cell lines growing in immune-deficient mice, chemically-induced (*e.g.,* 7,12-dimethylbenzanthracene; N-nitrosomethylurea) models in rats and in mice (usually with progestin priming), virally-induced (*e.g.,* mouse mammary tumor virus; polyomavirus), and genetically manipulated mouse models. Each of these animal models has several limitations, which have been widely discussed elsewhere. For example, a viral etiology for human breast cancer has yet to be established; human breast cancer cell lines have adapted to cell culture and may not fully reflect the complex characteristics and heterogeneity of breast cancer. Genetically manipulated models are often driven by a single gene activated/eliminated in all mammary cells, an occurrence that may be relatively rare in the majority of sporadic (non-familial) breast cancers where a single driver gene may not consistently dominate. For example, estrogen receptor alpha and erbB2 signaling appear to dominate in some breast cancers but this dominance may change as the disease progresses, e.g., the development of resistance to drugs that target these molecules is well documented.

The chemically-induced rat models are among the most widely used in the experimental mammary cancer studies cited in this report. The endocrine responsiveness of the mammary gland in rats, and of the chemically-induced mammary tumors that arise in these rodents, are thought to mimic closely what also is known to occur in humans. This is likely for estrogens (MNU-induced tumors are usually more estrogen responsive than DMBA-induced tumors), and it appears also to be likely for insulin and the IGFs. One potential exception (and this applies to many rodent mammary tumor models) is their apparent prolactin dependency. It is not yet clear that prolactin plays such a central role in human breast cancers. With respect to the histopathology of rodent models, rat tumors exhibit significant similarities to early breast adenocarcinomas in women; this comparative physiology/biology has been extensively described *(492-494)*. The mammary tumors arise primarily in the terminal end buds, which are comparable structures to the terminal ductal lobular units in the human breast where most human breast cancers arise. Nonetheless, there are also notable limitations. For example, the chemicals used to induce the mammary tumors in rodents are generally not thought to be major environmental exposures in human populations. Approximately 20% of DMBA-induced mammary tumors, and almost 85% of MNU-induced mammary tumors, have Ha-ras mutations. The role of this mutation in human breast cancers has not been clearly established, although ~30% of human breast tumors exhibit ras overexpression that may or may not activate similar or closely related pathways to those seen in the rodent models.

The mouse is also a good rodent model in which to study normal mammary gland development, although for breast cancer studies some mouse mammary tumor models exhibit rare or unusual histopathologies with respect to the human disease. The estrogen responsiveness of some mouse mammary tumor models is perhaps not seen as consistently as it is in the rat models; some mouse models generate ER+ mammary tumors that do not exhibit estrogen sensitivity and so may be models of those ER+ breast cancer that are *de novo* resistant to antiestrogens and aromatase inhibitors. There are certainly notable exceptions, e.g., some chemically induced mouse models that arise with DMBA following progestin priming and

some virus-induced models. Genetically manipulated mouse models (often tissue-specific) that show an endocrine responsiveness similar to human breast cancer are also beginning to appear in the literature.

None of the rodent models is ideal for establishing molecular mechanisms immediately relevant to humans but they appear effective in establishing hypotheses for further exploration in the human disease. Nonetheless, the receptor systems most closely associated with human breast cancers and their respective functions seem similar and rodents appear to be reasonable models in which to study the molecular mechanisms of endocrine effects on mammary tumorigenesis. As such, the rodent mammary tumor models represented in studies cited in this project seem to be, in principle, adequately suited to this limited purpose.

A large number of animal studies have been conducted to assess the impact of soy isolate or individual isoflavones on mammary gland development and carcinogenesis. Tomar and Shiao, 2008 *(479)*, funding support not indicated but authors affiliated with the Office of Environmental Health Hazard Assessment, California Environmental Protection Agency, published a literature review on early life and adult exposure to isoflavones and breast cancer risk. This review included an assessment of almost 40 studies conducted in rats and mice. In the 25 rat studies, the majority used an experimental design where Sprague-Dawley rats were exposed to soy diets, soy extract, or individual isoflavones and then subsequently treated with the chemical carcinogen 7,12-dimethylbenz[α]anthracene (DMBA) or N-methylnitrosourea (NMU) to assess susceptibility to develop mammary gland tumors. Tomar and Shiao, 2008 *(479)* summarized these studies as usually showing borderline statistically significant effects of soy protein isolate or individual isoflavones on the risk to develop chemically-induced tumors. The studies rarely reported reductions in tumor incidence; instead the findings suggestive of a protective effect were increased latency to develop tumors or a reduction in tumor weight or volume, although the effects did not always show a clear dose-response. A review by Trock *et al.*, 2006 similarly concluded that genistein exposure starting with co-exposure to a carcinogen or when mammary tumors are palpable does not decrease mammary tumor incidence or multiplicity but may increase tumor latency (Cohen 2000, Ueda 2003, Jin 2002, Constantinou 2001, Gallo 2001 as reviewed in Trock *et al.*, 2006 *(485)*) .

The studies that assess the impact of early in life exposures are discussed in detail in Chapter 3, but the underlying hypothesis is that undifferentiated mammary gland structures such as terminal end buds are generally considered more susceptible to mammary gland carcinogenesis and exposures that delay the maturation of these structure may cause an enhanced response to subsequent treatment with DMBA or NMU, often administered during early adulthood, e.g., PND 50 *(479; 492)*. The degree of maturation is typically assessed by measuring the number or density of undifferentiated and differentiated structures. The converse scenario is that exposures that accelerate the maturation of mammary gland structures may have a protective effect on mammary tumor development. The Tomar and Shiao, 2008 review *(479)* concluded that any protective effects of soy or isoflavones were mostly shown following exposure late in the juvenile period or adult Sprague-Dawley rats. A protective effect was not observed in animals following fetal with continuous exposure in adulthood. Tomar and Shiao noted that species differences in the production of equol from daidzein between rats (100% equol "producers") and humans (~20-40% equol "producers") complicates extrapolation of the rodent findings to humans.

In contrast, Tomar and Shiao *(479)* concluded that isoflavones show estrogenic effects on tumors that are already formed or in transgenic mouse models with oncogenes. This conclusion was primarily based on 12 studies conducted in mice. In these studies, the animal model was often a transgenic

mouse, e.g., activated MMTVneu/ErbB-2 oncogene, ERαKO, or ovariectomized mice implanted with breast cancer cells, e.g., MCF-7, MDA-MB-435, or F_3II. The studies that involve ovariectomized animals implanted with cells from a breast cancer cell line are considered models for post-menopausal women. Tomar and Shiao, 2008 reported that three of the studies showed a protective effect, six showed a negative effect, and the remaining observed no effect of isoflavones on tumor incidence.

Messina and Wood, 2008 *(490)*, Duffy *et al.*, 2007 *(481)*, and Trock *et al.*, *(485)* also reviewed rodent studies that looked at the effect of isoflavones on breast tumor growth. Some studies reported that isoflavones stimulate the growth of ER+ human breast cancer cell xenografts in ovariectomized athymic mice *(495)* (Hsieh 1998, Ju 2001, Allred 2001a, Allred 2001b, and Allred 2004 as reviewed in Messina *et al.*, 2008 and Duffy *et al.*, 2007 *(481; 490)*), stimulate estrogen-dependent mammary tumors in rats (Allred 2004 as reviewed in Messina and Wood, 2008 and Duffy *et al.*, 2007 *(481; 490)*), and that genistein can inhibit the efficacy of tamoxifen in suppressing the growth of MCF-7 cells implanted in athymic mice or increase tumor multiplicity in rats given DMBA and co-treated with genistein and tamoxifen compared to tamoxifen alone (Ju 2002 and Constantinou 2005 as reviewed in Messina and Wood, 2008 and Duffy *et al.*, 2007 *(481; 490)*). Genistein is the isoflavone considered to be primarily responsible for the tumor stimulating effects and some research indicates that the more processed soy products cause faster tumor growth compared to products that are less processed (Allred 2004, Ju 2006 as reviewed in Messina and Wood, 2008 and Duffy *et al.*, 2007 *(481; 490)*). However, other rodent studies reported that treatment with isoflavones, soy protein, or genistein suppressed tumor growth of MCF-7 or other breast cancer cell line implanted in mice and/or enhanced the suppressive effect of tamoxifen on tumor growth (Hewitt 2003, Shao 1998, Constantinou 2001, Zhou 2004, Hawrylewicz 1995, Mai 2007, Yan 2002 and Gotoh 1998 as reviewed in Messina and Wood, 2008 and Duffy *et al.*, 2007 *(481; 485; 490)*).

The apparently mitogenic effects of genistein or soy exposure in xenografts are most consistent with the data that concentrations of genistein achievable *in vivo* appear almost exclusively mitogenic when added to estrogen-dependent human breast cancer cells growing *in vitro*. Indeed, a large number of *in vitro* studies show that genistein has proliferative effects on MCF-7 cells, perhaps the most widely used ER-positive/estrogen-dependent human breast adenocarcinoma cell line. Fan *et al.*, 2006 *(496)* reported that low concentrations of genistein (0.5–1.0 μM can induce expression of the breast cancer susceptibility genes BRCA1 and BRCA2 in human cells lines for breast cancer (MCF-7 and T47D) and prostate cancer (DU-145 and LNCaP), possibly by activating endoplasmic reticulum stress response signaling. The authors conclude these activities suggests a potential relevance to cancer prevention because both BRCA1 and BRCA2 have been identified as tumor suppressors for breast and prostate cancer and the loss of BRCA1 expression or function is linked to breast cancer. However, the unfolded protein response induced by endoplasmic reticulum stress can be both prosurvival and prodeath. In breast cancer cells, estrogenic signaling induces a key component of the unfolded protein response (XBP1 and its splicing by IRE1-alpha), which can confer both estrogen-independence and resistance to antiestrogens *(497; 498)*These activities are prosurvival and appear more consistent with the reported estrogenic (and mitogenic) effects of genistein in ER+ breast cancer cells. Genistein's ability to inhibit protein tyrosine kinases, involved in growth signaling pathways, is a proposed mechanism to account for reports that isoflavones may inhibit proliferation in hormone-independent breast cancer cell line (reviewed in *(481)*). However, these effects appear to occur at concentrations that may not be easily achieved *in vivo (489)*.

2.4.3 Prostate Cancer

The highest incidence rates for prostate cancer are reported for men in the United States, Australia, and Canada. In contrast, some of the lowest incidence rates are reported in Asian countries such as Singapore, Japan, China and Hong Kong *(499)*. Similarly, deaths due to prostate cancer (per 100,000 population) are greater in Western countries, such as Norway (28.4), United Kingdom (17.9), and the United States (15.8) compared to Japan (5.7) and China (1.0) (Jemal *et al.*, 2006 as reviewed in Goetzl *et al.*, 2007 *(499)*). In addition, estrogen-based therapies have been used to treat prostate cancer because they can act to achieve a state of hormonal castration where the estrogen-based therapy can reduce the release of luteinizing hormone from the pituitary gland, resulting in a reduction in testicular production and release of testosterone. In addition to the estrogenic effects of certain isoflavones, other mechanisms that have been implicated in relation to prostate carcinogenesis include cell cycle inhibition, anti-angiogenesis, and induction of apoptosis (reviewed in Goetzl *et al.*, 2007 *(499)*). For these reason, a large number of studies have evaluated the association between exposure to soy foods and/or isoflavones and prostate cancer risk.

Yan *et al.*, 2009 *(500)*, supported by the USDA Agricultural Research Service, conducted a meta-analysis of 15 epidemiological publications on soy consumption (6 cohort and 9 case-control) or isoflavones (2 cohort and 7 case-control) in association with prostate cancer risk. One of the soy food studies and one of the isoflavone studies were excluded because they were earlier or identical versions of other published studies; thus, only 14 studies were considered for soy foods and 8 for isoflavones. The analysis of 14 studies on soy intake yielded a combined RR/OR of 0.74 (95% CI: 0.63 - 0.89; *P*=0.01). The authors also performed a separate analysis of the 14 studies to see if there were differences between the 8 that provided data on non-fermented food, i.e., soy milk and tofu, and the 6 on fermented food, i.e., miso and natto. Studies on non-fermented soy foods yielded a combined RR/OR of 0.70 (95% CI: 0.56 - 0.88; *P*=0.01) and those on fermented soy foods yielded a combined RR/OR of 1.02 (95% CI: 0.73 - 1.42; *P*=0.92). The analysis of studies on isoflavones yielded a combined RR/OR of 0.88 (95% CI: 0.76 - 1.02; *P*=0.09). The studies on isoflavones were also separately analyzed on the basis of whether they were conducted in Asian, Europe, or the U.S. The analysis from studies in Asian populations showed a combined RR/OR of 0.52 (95% CI: 0.34 - 0.81; *P*=0.01) and from studies with Western populations a combined RR/OR of 0.99 (95% CI: 0.85 -1.16; *P*=0.91). The authors concluded that the available epidemiologic studies suggest that consumption of soy foods is associated with a reduction in prostate cancer risk in men and that this protection is related to the type and quantity of soy foods consumed.

Balk *et al.*, 2005 *(80)* reviewed 3 randomized trials and 2 cohort studies, considered to be of generally poor quality, that investigated the effect of soy protein or pure soy isoflavones on testosterone levels in healthy males. Higher testosterone is considered a potential risk factor for prostate cancer by the NCI; therefore, any beneficial effect of soy would be expected to decrease testosterone levels. Balk *et al.*, 2005 concluded that the limited evidence suggests a possible trend to lower risk for prostate cancer, with consumption of soy products by men. However, there was insufficient evidence regarding different types or doses of soy products to compare their relative effectiveness.

Van Patten *et al.*, 2008 *(501)*, supported by the Michael Smith Foundation for Health Research, reviewed 9 randomized controlled trials of diet or dietary supplements in men with prostate cancer using prostate specific antigen (PSA) or PSA doubling time as a serum biomarker of prostate cancer recurrence and/or survival. Phytoestrogens were the most studied dietary intervention in men with

prostate cancer and was a factor in 7 of the reviewed studies Overall, the results were mixed and phytoestrogens in the diet were not associated with a consistent direction of effect on PSA or PSA doubling time. Goetzl *et al.*, 2007 *(499)* also concluded that the results from existing clinical trials are mixed and do not provide sufficient evidence for a chemoprotective effect of soy isoflavones on the development of prostate cancer. However, they noted that a number of phase I, II, and III randomized trials were listed in the NIH clinical trial database (*http://www.ClinicalTrials.gov*) designed to address the impact of soy foods of isoflavones on prostate cancer-related endpoints, including PSA, biomarkers of cell cycle regulation, PIN score, and tumor progression.

Most experimental animal studies on soy isoflavones and prostate cancer were conducted in rodents with implanted tumors or chemically induced cancers, but a limited number of studies were conducted in genetically susceptible strains *(3)*. This is because rodents rarely develop prostate cancer naturally. In most rodent studies, genistein or isoflavones were found to inhibit prostate tumor growth (reviewed in *(3; 502)*). For example, Pollard and Suckow, 2006 *(503)* published a review of several studies where in Lobund-Wistar rats were fed a soy-containing diet to see if the development of spontaneous of MNU-induced prostate cancer was altered. The Lobund-Wistar rats is a rodent strain that has high testosterone levels and are predisposed to developing spontaneous hormone-refractory prostate cancer (HRPC) and developing MNU- induced prostate tumors. Based on a review of six studies, the authors concluded that the administration of a soy-containing diet to the Lobound-Wistar rats prevented spontaneous and induced tumors and led to moderate reductions in testosterone levels. Cooke, 2006 *(254)* published a review on animal models used to investigate the effects of soy isoflavones on prostate cancer (Pollard and Wolter, 2000, Dalu *et al.*, 1998, Hikosaka *et al.*, 2004, Cohen, 2002, Zhou *et al.*, 1999, Mentor-Marcell *et al.*, 2001, and Mentor-Marcell *et al.*, 2005 as reviewed in Cooke, 2006 *(254)*). Cooke concluded that the animal studies, conducted in rats or mice, indicated a protective effect during the androgen-responsive state of tumor development but after this stage, isoflavones are ineffective and may be agonists.

2.4.4 Colon Cancer

According to the UK Committee on Toxicity *(3)*, human studies generally have indicated an increased risk of colorectal cancer with consumption of fermented soy products and a decreased risk of colorectal cancer with consumption of non-fermented soy products. Most of the human studies were conducted with soy products, and the UK Committee on Toxicity noted the possibility that another active component in soybeans contributed to observed effects.

Investigations of possible links between genistein and colon cancers are limited to experimental animal studies, and these studies reported conflicting findings (reviewed in *(3; 502)*). The UK Committee on Toxicity *(3)* concluded that experimental animal studies provided some evidence of beneficial effects of phytoestrogens on breast and prostate cancer but were inconclusive for colon cancer. Similarly, Cassidy *et al.*, 2006 *(145)* concluded that the available evidence shows that the consumption of soybean does not lead to a reduced risk of colorectal cancer.The committee conclusions are in contrast to those of Kurzer and Xu (179), who reported that there is much epidemiological evidence to support the hypothesis that isoflavones can reduce the risk of breast, colon, and prostate cancer.

Cooke, 2006 *(254)* reviewed 6 studies on genistein or dietary soy isoflavone and colon cancer conducted in rats and mice, including an induced male rat model, ERαKO mice, and athymic mice (Thiagarajan *et al.*, 1998, Davies *et al.*, 1999, Vis *et al.*, 2005, Kallay *et al.*, 2002, Guo *et al.*, 2004,

and Gentile *et al.*, 2003 as reviewed in Cooke, 2006 *(254)*). Findings included reduced number of foci with aberrant crypts, no reduction in tumor frequency or volume, decreased tumor incidence following chemical induction, increased fecal fat excretion, and maintainence of vitamin D levels in the colon. The overall conclusion of Cooke, 2006 based on the animal studies was that soy isoflavones may provide some protection against the progression of colon cancer.

2.5 Potentially Sensitive Subpopulations

Studies in humans identified inter-individual differences in toxicokinetics and metabolism of isoflavones, possibly due to variations in gut microflora activity. In a study by Zhang *et al.*, 1999 *(197)*, fecal microflora degradation was investigated in 25 volunteers. Among those volunteers, the study authors identified 7 males and 7 females whom they classified as having moderate degradation activity. The authors speculated that less fecal activity would result in reduced degradation of isoflavones, leading to increased blood levels and urinary excretion. In those individuals, mean half-lives for fecal degradation were 8.9 ± 4.3 hours for genistein and 15.7 ± 5.3 hours for daidzein. Half-life ranges in these individuals were 4.0–16.8 hours for genistein and 5.3–23.2 hours for daidzein. **[The Expert Panel notes the wide range of activity among volunteers. The study authors admitted that they had to include a few volunteers with relatively long or short half-lives in order to get a sufficient number of subjects for this study.]** Despite attempting to select subjects with similar fecal degradation rates, the study authors noted a high rate of variation for urinary excretion, with 8-fold differences noted for genistein, 5-fold differences for daidzein, and 4.5-fold differences for glycitein.

Metabolic capacity early in life

Certain polymorphisms in UGT1A1 are associated with lower glucuronyltransferase activity. For example, individuals with Gilbert's syndrome have higher serum levels of unconjugated biliburin due to a polymorphism in the promoter region of UGT1A1*28 (7/7 genotype for the $A(TA)_7TAA$ allele) that results in significantly lower bilirubin UDP-glucuronytransferase enzyme activity compared to individuals with a 6/6 genotype for the A(TA)6TAA allele *(504)*.

Presumably do to the differences in gut flora, it has been reported that bacterial β-glucosidase activity is lower in infants compared to adults and increases with age (reviewed in *(6)*) **[indicating that absorption is likely to be lower in infants than adults]**.

As noted in **Section 2.1** on toxicokinetics, most genistein and daidzein is present in the circulation as glucuronide conjugates. Studies in humans suggest that infants may have a decreased ability to glucuronidate isoflavones because UDPGT activity is low in the fetus and neonate but gradually increases to adult levels in months to years (reviewed in *(216)*).

Coughtrie *et al.*, 1988 *(245)* examined the ontogeny of UDPGT in humans. Activity was measured in postmortem liver microsome samples obtained from adults and premature or full-term infants. Results of this analysis are listed in **Table 83**. Activities for isoenzymes catalyzing glucuronidation of bilirubin, testosterone, and 1-napthol were very low at birth in premature and full-term infants. Activities increased with age for the isoenzymes catalyzing glucuronidation of bilirubin, primarily UGT1A1 (~80% of adult levels by 8–15 weeks of age) and 1-naphthol (~30% of adult levels at 8–15 weeks of age). During the first 55 weeks of life, no consistent increase in activity was noted for the isoenzyme catalyzing glucuronidation of testosterone. Using an immunoblot technique with antibodies

Table 83. Development of UDPGT activity in Humans (Coughtrie et al., 1988)

Age	*n*	*UDPGT Activity towards Each Substrate, nmol/min/mg protein*		
		Bilirubin	*Testosterone*	*1-Napthol*
30 weeks gestation		0.05	0	0.56
30 weeks gestation with 10 weeks survival		0.4, 1	0.14, 0.85	3.0, 1.8
Full-term infants surviving 1 – 10 days	7	0.07 ± 0.04	0.10 ± 0.06	0.75 ± 0.68
Full-term infants surviving 8 – 15 weeks	6	0.64 ± 0.32	0.12 ± 0.05	2.4 ± 1.1
Full-term infants surviving 22 – 55 weeks	5	0.99 ± 1.1	0.09 ± 0.06	3.6 ± 2.1
Adult males	3	0.76 ± 0.43	0.46 ± 0.61	7.2 ± 2.2

Data presented as individual values or mean ± SD.
From Coughtrie et al., 1988 *(245)*.

developed toward liver testosterone/4-nitrophenol and kidney naphthol/bilirubin, 1 immunoreactive protein was observed in microsomes of 18- and 27-week-old fetuses, 3 immunoreactive proteins were observed in microsomes of term infants, and most isoenzymes present in adults were observed within 3 months of age at levels ~25% those of adults.

Despite the possibility of lower UDPGT activity in infants, a letter to the editor providing few details except a reference for the analytical method used reported no detectable levels of unconjugated isoflavones in plasmas from 4 infants (2.5–5.5 months old) fed exclusively soy formula for at least 2 weeks *(505)*; blood samples had been measured before and after hydrolysis with β-glucuronidase and sulfatase, but the percentages of each conjugate were not specified. **[The Panel was not able to verify this information due to lack of experimental details and data. This reference is presented for completeness and will not be considered further.]**

Coughtrie *et al.*, 1988 *(245)* also measured activity and expression of UDPGT in hepatic microsomes of WAG rats from GD 17 to PND 75. Consistent results were obtained using methods to measure enzyme activity and protein levels via immunoreactive probes. Activity of the isoenzyme catalyzing the glucuronidation of testosterone was barely detectable in fetuses, increased to ~20% of adult levels at birth, and continued to increase until reaching adult levels between 26 and 30 days of age (with the exception of a decrease on PND 40). Activity of the isoenzyme catalyzing glucuronidation of bilirubin was barely detectable in fetuses, increased at birth to reach 75% of adult levels on PND 2–16 (with the exception of a decrease on PND 5), and reached or exceeded adult levels by PND 20 (with the exception of a decrease on PND 40). The isoenzyme catalyzing glucuronidation of 2-aminophenol had ~30–60% of adult activity in fetuses, reached or exceeded adult activity on PND 2–5, had ~30% of adult activity on PND 10–20, and reached or exceeded adult activity by PND 26. **[It is difficult, however, to predict liver UDPGT isoenzyme activity from gut, and vice versa. The Expert Panel noted that isoenzyme expression is tissue-specific *(506)*.]**

A study by Cotroneo *et al.*, 2001 *(215)* demonstrated that sc injection of rats with 500 mg/kg bw

genistein on PND 21, 50, or 100 resulted in blood genistein levels that were ~2 orders of magnitude higher on PND 21 than PND 50 or 100. **[The Expert Panel notes that the higher blood genistein levels on PND 21 indicate reduced clearance in immature rats. The finding has possible implications regarding accumulation of genistein and potential toxicity in immature rats.]**

Some sex-specific differences were observed in a study in which male and female rats were gavaged with 4 mg/kg ^{14}C-genistein *(195)*. Plasma levels of label were higher in males (C_{max}=2250 ng/mL, AUC=14,147 ng-h/mL) than females (C_{max}=601 ng/mL; AUC=8353 ng-h/mL), and half-life in males (12.4 hours) was longer than in females (8.5 hours). The major fecal metabolite was 4-hydroxyphenyl-2-propionic acid in males, but dihydrogenistein was the most abundant fecal metabolite in females. Radioactivity was higher in livers of females than males. While sulfated genistein was the most abundant compound in livers of males, parent genistein was the species measured at the highest concentration in livers of females.

A second study in rats also reported sex-related differences in toxicokinetics of genistein *(210)*. The rats were given feed containing genistein at 5, 100, or 500 ppm from weaning to PND 140. Compared to males, females had higher levels of total genistein in serum, liver, and mammary gland, a higher AUC, and a longer half-life. Complete details of this study and the apparent discrepancy between these 2 rat studies are discussed in **Section 2.1**.

Minimal sex-specific differences in neonatal female compared to male mice treated sc with genistein included higher C_{max} for total genistein, slower initial conjugation, and a major secondary peak of conjugated genistein in serum, indicative of enterohepatic cycling *(216)*.

Equol producers

As discussed in **Section 2.1.1.2**, the metabolic profile of daidzein varies among individuals. Some individuals produce little or no equol but approximately 30-50% of individuals are able to convert daidzein to equol ("equol producers") *(106; 172; 173; 190; 191)*. A growing literature is focused on assessing whether the ability of certain individuals to produce equol is a contributing factor in the variability in response observed in studies focused on potential health benefits of soy because equol has a higher estrogenic potency compared to daidzein *(142)*.

2.6 Summary of Pharmacokinetics and General Toxicology/Biological Effects

2.6.1 Pharmacokinetics

Genistein, daidzein, and glycitein exist mainly in their glycosidic forms in unfermented soy foods. Before isoflavone glycosides can be absorbed into the systemic circulation, they must first be hydrolyzed to their aglycones, which have greater hydrophobicity and lower molecular weight. Prior to systemic availability, most genistein and daidzein are conjugated with glucuronic acid by uridine diphosphate (UDP)-glucuronosyltransferases; a smaller amount is conjugated to sulfate by sulfotransferases. Conjugation of isoflavones can also occur in liver. The glucuronide and sulfate conjugates enter the systemic circulation, and the majority of isoflavone compounds in the circulation are present in conjugated form.

2.6.1.1 Humans

In humans, a considerable amount of pharmacokinetic information is available for genistein and daidzein in adults. Less information is available for equol and very little data has been published on

glycitein. Human developmental pharmacokinetic data are lacking; the available "pharmacokinetic" data in human infants essentially are equivalent to biomonitoring data.The detection of genistein, daidzein, and equol in serum, urine, amniotic fluid, cord blood, and breast milk in humans demonstrate fetal exposure and absorption into the systemic circulation of infants. Full pharmacokinetic data on genistein, daidzein and/or glycitein among human infants following soy formula feeding have not been published. Rather, data are limited to single plasma concentrations, generally after chronic soy formula feeding, which likely represent steady state (although this is uncertain) and spot urinary concentrations [84; 87; 91; 94; 95; 175; 199-202]. No studies of human infants fed genistein, daidzein and/or glycitein have been reported. Thus, the human pharmacokinetic data is solely based on studies conducted in adults. Data in infants and children are limited to biomonitoring data.

Relatively few studies include measurement of glycitein and it has not been measured in biomonitoring studies of the general population conducted by the CDC as part of NHANES *(115)* or measured in the plasma or urine of soy formula-fed infants *(84; 91; 94; 95)*. If glycitein is measured in humans, it is generally following intentional dosing of subjects with a soy protein or isoflavone supplement.

Prior to reaching the systemic circulation, most genistein and daidzein is conjugated to glucuronic acid by UDPGT; a much smaller amount is conjugated to sulfate by sulfotransferase enzymes *(3; 179; 180)*. Conjugation of genistein occurs in the intestine but also has been reported to occur in liver. Based on *in vitro* data, the enzymes most involved in glucuronidation are: UGT1A1, 1A8, 1A9, 1A10 *(187)* whereas the SULT enzymes likely responsible for sulfation include SULT1A1 and SULT2A1 *(77; 188)*. However, interpretation of the *in vitro* data requires information on tissue specific expression, particularly for human intestine and liver. Moreover, to inform the question of disposition of soy formula isoflavones, the ontogeny of such tissue specific expression is needed. Only a limited amount of such data are available *(189)* precluding estimates of developmental differences among humans, as well as interspecies differences among the young.

The glucuronide and sulfate conjugates can enter the systemic circulation, and it has been reported that the majority of isoflavone compounds in the circulation are present in conjugated form, thus limiting the bioavailability of the aglycone forms of isoflavones. In studies in which humans were exposed to genistein or isoflavone aglycones at genistein doses of 1–16 mg/kg bw, most of the genistein was present in plasma in conjugated form, while free genistein represented 1–3% of total plasma genistein levels in most cases *(76; 153; 168)*. Conjugated isoflavones undergo enterohepatic circulation, and on return to the intestine, they are deconjugated by bacteria with β-glucuronidase or arylsulfatase activity. The metabolites may be reabsorbed or further metabolized by gut microflora. Isoflavones can undergo further biotransformation that ultimately leads to the formation of 6′-hydroxy-*O*-desmethylangolensin from genistein and *O*-desmethylangolensin from daidzein *(3; 179; 180)*. The metabolic profile varies among individuals, with some individuals producing little or no *O*-desmethylangolensin or equol, an intermediate metabolite of daidzein that is biologically active.

Based on the summary of the studies presented in Nielsen and Williamson, 2007 *(143)* and Cassidy *et al.*, 2006 *(145)*, pharmacokinetic parameters (C_{max}, t_{max}, $t_{1/2}$, AUC) are similar for genistein and daidzein. For genistein, the average t_{max} was 5.7 hours (range of 3.5–9.3 hours) and for daidzein, it was 6.2 hours (range of 4.0–8.3 hours). These estimates are consistent with the range of peak values of ~6–8 hours reported by others for genistein and daidzein following ingestion of soy or isoflavones

(*(112)*; reviewed in *(1; 3; 108)*). The maximum concentrations (C_{max}) of genistein and daidzein, when normalized to ingestion of 1 µmol/kg body weight, were also similar at 0.49 or 0.64 µM or 0.50 or 0.54 µM, respectively. The average half-life of elimination ($t_{1/2}$) estimates from reviews by Nielsen and Williamson 2007 *(143)* and Cassidy *et al.*, 2006 *(145)* did not differ between genistein, 9.5 hours (range 6.1-17 hours), and daidzein, 7.7 hours (range 4.2–16 hours). These estimates are generally similar to those presented in a recent review by Larkin *et al.*, 2008 *(1)*; 3–9 hours for daidzein and 8–11 hours for genistein after intake of soy foods or pure isoflavone glycosides. The AUC values, when normalized to ingestion of 1 µmol/kg body weight, were 11 µmol*hour/L for genistein and 18 µmol*hour/L for daidzein. Absorption half-lives for both daidzin and genistin, presumably as glucuronides and sulfates of daidzein and genistein, were reported at ~1–3 hours following intake of foods containing ≤210 mg of each isoflavone or providing doses of ≤2 mg/kg bw/day of each isoflavone (*(164; 165)*; reviewed in *(108)*).

Studies in humans that report the bioavailability and other pharmacokinetic parameters of isoflavones ingested as glycosides versus aglycones have reported conflicting findings (reviewed in *(3; 143; 145)*). While there is no clear explanation for the conflicting findings on bioavailability of the aglycone and glucoside, one factor may be the differences in the type of isoflavone preparations administered to subjects, e.g., soybean extracts containing mixtures of isoflavones, purified single isoflavone, ingestion in tablet or liquid form *(142)*. In addition, comparing the conclusions on bioavailability across studies is difficult because of the variety of indices used to assess bioavailability, e.g., relative AUC, C_{max}, t_{max}, urinary excretion and recovery, etc. Prasain and Barnes *(146)* discussed the various approaches used to assess the bioavailability of isoflavones. In pharmacology (and toxicology) the term absolute bioavailability refers to the ratio of AUC after oral ingestion to the AUC after systemic administration, i.e., IV injection. This is the fraction of the compound absorbed through non-intravenous administration compared with the corresponding intravenous administration of the same drug. This approach may underestimate bioavailability at the tissue level if circulating isoflavones in the conjugated form are converted to aglycones by β-glucuronidases and sulfatases secreted by cells within target tissues. In the nutrition literature, bioavailability is often assessed by the percent of administered isoflavone that is recovered in the urine. Higher degrees of recovery in urine are interpreted as more bioavailability based on the assumption that the isoflavone must have been present in blood prior to reaching the kidney and ultimately being excreted in urine.

In their review, Nielsen and Williamson, 2007 *(143)* concluded that despite the apparently contradictory findings, the data are consistent enough to conclude that (1) at equivalent doses, the C_{max} is higher for genistein and daidzein following administration as glucosides compared to aglycones, and (2) the half-life is not significantly different for aglycone and glucoside. In addition, they concluded that deglycosylation is required for absorption but does not appear to be a rate-limiting step. As noted above, studies by Setchell *et al.*, 2001 *(76)* and Rufer *et al.*, 2008 *(166)* suggest the percent of genistein and daidzein circulating in the unconjugated forms do not differ based on administered form. These conclusions support the assumption used by others that because glycosides are quickly deconjugated in the gut to form the active aglycones, exposure to a particular isoflavone (e.g., genistein) is theoretically the sum of the aglycone and respective glycoside compound concentrations converted on the basis of molecular weight *(3; 5; 6; 17)*.

No pharmacokinetic studies, defined as measurement of isoflavone concentrations in serial blood or plasma samples, have been conducted in infants or children. The only data that are interpretable in this

context are the data presented in Setchell *et al.*, 1997 *(84)* and Cao *et al.*, 2009 (*(94)*). These studies reported genistein and daidzein concentrations in plasma or blood samples obtained at unspecified times relative to feeding in infants chronically fed soy formula. Usual feeding behavior was reported to result in mean genistein concentrations of 684 ng/ml and 757 ng/ml, and mean daidzein concentrations of 295 ng/ml and 256 ng/ml by Setchell *et al.*, 1997 *(84)* and Cao *et al.*, 2009 *(94)* respectively. Data obtained by Cao *et al.*, 2009 *(94)* indicated that total genistein concentrations in whole blood ranged from approximately 13.5–3562.9 ng/ml (range data obtained from personal communication with Dr. Cao, December 2009). Assuming that the infants were consuming soy formula for more than 2-3 days, these values approximate a steady state condition, and the reported concentrations provide a context in which animal data can be prioritized and interpreted.

Limited data suggest low levels of equol are detected in both whole blood and urine of infants, regardless of type of feeding *(6; 84; 87; 94)*. Setchell *et al.*, 1997, 1998 *(6; 84)* detected equol in all cow-milk formula-fed infants, 4/7 soy formula-fed infants and 1/7 breast-fed infants. In contrast, Cao *et al.*, 2009 *(94)* did not detect equol in the blood of any infant, but reported detection of equol in urine among infants fed cow milk formula (22%) compared to those fed soy formula (5%) or breast fed infants (2%). Also, given that equol is present in breast milk and cows milk *(6; 71)*, it does not appear that soy-based formula is associated with increased exposure to equol relative to cow milk-based formula or breast milk. Collectively, these data indicate that relatively low exposures to equol can be detected in infants fed soy formula, but also in infants fed cow milk-based formula and breast milk. Thus, this level of equol exposure in human infants is markedly lower than that reported in animals fed soy-based diets or daidzein. These data from human infants provide a context for interpreting the animal data presented in **Chapter 3.**

2.6.1.2 Experimental animals

Genistein is absorbed in rats and mice following oral or sc exposure. According to data in **Table 38,** maximum genistein levels in blood are obtained within 2 hours of exposure. A mass-balance study of rats gavaged with ^{14}C-genistein 4 mg/kg bw reported V_d at 1.27–1.47 L *(195)*. **[The Expert Panel noted that the reported V_d suggests that most of the circulating radioactivity was not genistein but the glucuronide.]** Plasma protein binding ranged from ~80 to 90%. Radioactivity was distributed throughout the body, with levels in reproductive organs (vagina, uterus, ovary, and prostate) higher than levels in other organs (brain, fat, thymus, spleen, skeletal muscle, and bone). Some studies demonstrated higher levels of genistein aglycone versus conjugates within tissues compared to blood, raising the possibility of accumulation or hydrolysis of aglycones within tissues *(77; 209; 210)*. **[The Expert Panel noted that differences between free genistein levels in blood and tissues is probably due to differences in how the aglycone and glucuronide compounds partition between fat in blood and tissues.]**

There are two high quality studies that together address the impact of route of administration in young mice (Doerge *et al.* 2002; Jefferson *et al.*, 2009). Based on these reports, the dose-adjusted AUC after oral genistin in CD-1 mice is about half that of the subcutaneous genistein. Although there is some inconsistency in the C_{max} data, both values are relevant to humans as they are within the range of blood and plasma concentrations reported in human infants.

Studies in adult rats and mice fed soy-containing feed reported that blood total equol levels were 2–10 times higher than blood total genistein and daidzein levels *(236; 238; 247)*. In most cases, total equol

levels were lower than or within the same order of magnitude as genistein and daidzein levels in fetuses or pups of rat dams fed soy-based diets *(236; 238)*. The half-life for genistein and its metabolites following ingestion of soy products by rats was reported at 8.8 hours. No data were found for half-life of daidzein and its metabolites following ingestion through soy foods. Urinary levels of total genistein and daidzein were reported to be ~25% of total equol levels in mice and ~50% of total equol levels in rats.

Studies demonstrated placental transfer of genistein to the rat fetus *(208; 209; 240)* and lactational transfer to the rat pup following dietary administration of genistein to the dam *(210)*. A study examining placental transfer reported higher concentrations of aglycone in fetuses compared to dams, leading the authors to conclude that placental transfer probably involves the aglycone; the finding was said to be consistent with limited conjugation ability of the fetal rat *(208)*. One study reported that the percentage of free genistein in milk from the pup stomach (78–97%) was higher than in milk from the dams' nipples (57%), suggesting that genistein conjugates may be hydrolyzed in the pup stomach *(209)*.

Studies in rats demonstrated the distribution of isoflavones and metabolites to fetuses during pregnancy or pups during lactation following ingestion of soy-containing feed by the dam *(236; 238)*. In one study, GD 20.5 fetuses and PND 3.5 pups contained about half the total genistein concentration and about one-tenth the total equol concentration measured in dams; total daidzein concentrations were about one-third lower in GD 20.5 fetuses and about the same in PND 3.5 pups compared to dams *(236)*. A second study reported that in pups born to dams fed a soy-containing diet, total genistein levels remained steady between birth and PND 12, while total daidzein levels were reduced by half during the same time period *(238)*. On PND 16, the time when pups likely began eating food, there was a 5- to 10-fold drop in total genistein and daidzein concentrations compared to earlier time periods. In contrast, total equol levels in pups decreased steadily following birth.

Genistein glucuronide is the most abundant genistein metabolite in rat blood *(195)*. Genistein is conjugated with glucuronide in the intestine and liver, and a study in rats demonstrated that the majority of glucuronidation most likely occurs in the intestine *(246)*. With the exception of 4-hydroxyphenyl-2-propionic acid, all other urinary genistein metabolites identified in rats were also reported for humans, suggesting pathways common to the two species. Parent compound was the predominant form of genistein in the uterus, while in prostate the most abundant form was the metabolite 4-hydroxyphenyl-2-propionic acid. One study reported no evidence that genistein aglycone or conjugate levels in blood were saturated following exposure to dietary genistein at up to 1250 ppm.

[The Expert Panel noted that comparisons of serum aglycone levels in adult versus fetal or neonatal rodents of the same study can be made from the rat data presented in Table 38 . A sc dosing study conducted in rats demonstrated similar percentages of serum aglycone (35–46%) at PND 21, 50, or 100. One study with gavage exposure demonstrated higher aglycone percentages in fetuses (27–34%) than dams (5–18%) on GD 20 or 21 *(208)*. A dietary study in which dams were fed 25 or 250 ppm genistein did not consistently demonstrate higher percentages of aglycone in dams (1.7–23%) compared to pups on PND 7 (14–19%) or PND 21 (6.6–33%) *(209)*. In an evaluation of all the data in Table 4, it was noted that percentages of free genistein following oral exposure of adult rats were usually below 10% but sometimes attained levels of ~20%; percentages of aglycone following direct and/or indirect oral exposure to genistein in rat pups ≤21 days old were reported at 1–33%.].

In a mass-balance study of rats gavaged with 4 mg/kg bw ^{14}C-genistein, ~65% of the dose was excreted in urine and 33% in feces at 166 hours following dosing *(195)*. About 90% of the dose was recovered within the first 48 hours following dosing. Total clearance was 1.18 mL/minute in males and 2.0 mL/minute in females. Genistein elimination half-lives have been reported at 2–9 hours in rats and 5–8 hours in mice *(195)*). **[The Expert Panel noted an apparent contradiction between the half-lives reported by Chang et al., 2000** (210) **(~3–4 hours) and Coldham and Sauer** (195) **(~9–12 hours). The differences in half-lives may have resulted from dosing regimen. Coldham and Sauer used a single low dose of 4 mg/kg bw and Chang et al., 2000 used a high daily dose rate of 50 mg/kg bw. The greatly decreased half-life at the higher dose may have resulted in part from saturation of glucuronidation and, hence, reduced enterohepatic circulation. Because it is expected that protein binding is saturated at high genistein doses, a much smaller portion of the higher dose would be bound to plasma proteins, contributing to the shorter half-life.]** In neonatal mice, elimination half lives were reported at 12–16 hours for genistein aglycone and 16–19 hours for genistein conjugate.

3.0 DEVELOPMENTAL TOXICITY OF SOY FORMULA

The Expert Panel's approach to considering the potential developmental toxicity of soy formula was to separately assess the literature for the individual isoflavones found in soy formula, i.e., genistein, daidzein (and its metabolite, equol), or glycitein) and the literature for mixtures of isoflavones in studies that assessed developmental effects from exposure to soy formula, soy diets, or other types of isoflavone mixtures.

Studies contained in Chapter 3 were categorized by the Expert Panel as being of "no," "limited," or "high" utility. Only those studies of "limited" or "high" utility are considered in preparing summary conclusions on the developmental toxicity of soy formula.

The Expert Panel's approach to considering the effects of soy formula on infant growth and development was to assess the literature for exposure to soy formula in infancy, using studies that described the following endpoints:

- Growth and nutrition
- Gastrointestinal effects
- Allergy and immunology
- Thyroid function
- Reproductive endpoints
- Cholesterol
- Diabetes mellitus
- Cognitive function

In assessing the aforementioned endpoints, the following criteria were used to produce the study strength and weakness statements and the overall assessment of the study utility for this report:

(1) Study Design:
   a. Use of appropriate controls
   b. Definition of primary and secondary endpoints
   c. Appropriate statistical analysis
   d. Longitudinal versus cross-sectional
   e. Randomization of infants to feeding groups

(2) Exposure to soy formula: Exclusive exposure to soy formula had to occur during infancy, i.e., exposure beyond 12 months was not considered.

(3) Follow-up

(4) Sample size: The adequacy of the study sample size was assessed based upon the endpoints of interest and their variability observed in the study such that statistical power could be determined. In general, longitudinal studies with fewer participants had greater justification for smaller sample size than the cross-sectional studies.

(5) Exposure assessment (in order of preference):
   a. Biochemical measurement of exposure
   b. Prospective
   c. Retrospective

(6) Control for confounding factors

The Expert Panel's judgement of "no", "limited" and "high" utility took into account each of the above factors.

The interpretation of many animal studies was hampered by the use of single dose levels, particularly when those dose levels were well above levels relevant to humans, use of treatment time periods that extended beyond development, the lack of reporting of litter data, and the lack of litter-based analysis. Route of exposure was a potentially important issue in the interpretation of studies. A working group of the expert panel met on June 8-9, 2009 to consider the impact of factors such as these in developing a consistent strategy for preparing strengths/weaknesses and utility statements for the experimental animal studies described in Chapter 3 of the draft Expert Panel report on Soy Formula. The following guidelines were developed by the working group:

(1) Lifestage of Exposure

Most weight in the assessment was given to those studies that exposed developing animals to soy, or in most cases the specific isoflavone genistein, solely during the period from birth to weaning because this window of exposure best approximates the timing of soy formula ingestion in infants.

(2) Route of administration

The oral route of dosing is considered most relevant for humans because exposure to the isoflavones found in soy formula occurs through the diet. The expert panel tried to preferentially evaluate studies that used oral administration since it best mimics the human route of exposure. However sc injection studies, particularly those using doses which produce blood levels representative of those measured following human exposure were also considered.

(3) Control for litter effects

Failure to use litter as the experimental unit in data analysis when this is appropriate is an important design weakness for gestational exposure studies, but did not necessarily result in a study being considered of "no" utility.

(4) Sample size

No pre-determined number was set as a cut-off for acceptable sample size. This decision will depend on the endpoint and animal model being assessed. For example, studies using sophisticated techniques, addressing specific experimental questions, or those that use primates may be justified in use of relatively small sample size.

(5) Single exposure level studies

Use of a single exposure level is not necessarily a study weakness and depends on the rationale for not utilizing multiple exposure levels.

(6) Use of positive control

Lack of a positive control is not counted against a study, but a negative study with a "failed" positive control for expected specific effects, e.g., estrogenic, will be considered of "no utility."

(7) Number of endpoints assessed

It is not necessarily a study weakness if only one or a small number of endpoints are assessed. This could reflect investigator's interest in a specific tissue and/or mechanism of action.

3.1 Human Studies on the Individual Isoflavones Found in Soy Formula

No human data were identified.

3.2 Human Studies of Soy Formula during Development

3.2.1 Exposure of infants

Reports on the ability of soy formula to support normal growth and to provide adequate nutrition are presented. Although several of these reports included premature infants, soy formula is not currently recommended for premature infants.

A number of studies have compared growth or tolerance in infants fed soy formula or other forms of soy-based nutrition, e.g., maize-soy flour, to infants fed a variety of alternatives to breastmilk besides cow's milk-based formula, including chicken-based formula, soy-formula supplemented with docosahexaenoic acid and arachidonic acid, or nutrient and calorie dense fortified spreads *(31; 507; 508)*. These types of studies are only summarized in the expert panel report on soy formula only if they included comparison with infants fed breastmilk or cow's milk-based formula.

3.2.1.1 Growth and Nutrition

Callenbach et al., 1981 ***(509)***, support not indicated, reviewed the records of 65 surviving infants who were born weighing less than 1500 g and for whom radiologic and biochemical data were available. There were 37 infants with radiologic evidence of rickets and 28 infants without such evidence. Soy-isolate formulas had been given to 32 of the children with rickets and 26 of the infants without rickets [P=0.69, Fisher exact test]. The authors observed that these infants were very ill, and that long periods of parenteral feeding had been required during the initial weeks of life.

Strengths/Weaknesses: Strengths include the focus on rickets and the evaluation of a homogenous special population of very low birth-weight infants with radiologic findings. Weaknesses include the retrospective nature of the record review, the lack of adjustment for confounders, and the small amount of variability in exposure: it appears that 58 of 65 infants in the sample had been given soy formula. The length of parenteral nutrition will largely influence the incidence of rickets or metabolic bone disease.

Utility (Adequacy) for CERHR Evaluation Process: This paper has no utility in the evaluation process.

Chan et al., 1987 ***(510)*****,** supported by Ross Laboratories, randomized 10 male and 10 female term newborns per group to receive 1 of 2 soy formulas that differed only in carbohydrate source (100% glucose polymers compared to 50% glucose polymers + 50% sucrose). A comparison group of 5 male and 5 female newborns who were exclusively breast-fed was used for comparisons up to 4 months of age, and a historical control group was used for comparisons at 6 and 12 months. Breast-fed infants were given supplemental vitamins and fluoride. Weight, length, and head circumference were determined at 2 weeks, 2 months, and 4 months of age, and serum was obtained for total calcium, phosphorus, magnesium, 25-hydroxycholecalciferol (a D-vitamin precursor), and alkaline phosphatase. Plasma and erythrocyte copper and zinc were measured, and bone mineral content (bone mineral density/bone width) of the distal radius was measured **[technique not specified, but probably single-photon absorptiometry, which was in widespread use during the time period].** No differences were detected between groups in any of the measures of growth or serum

chemistries. Plasma but not erythrocyte zinc was higher in breast-fed than formula-fed infants at 4 months (mean ± SD 74 ± 11 µg/dL in the breast-fed group compared to 63 ± 15 and 59 ± 6 µg/dL in the formula-fed groups). Bone mineral content was higher in the breast-fed group than in the formula-fed groups at 2 and 4 months. **[Mean bone mineral content was estimated from a graph: 2 months 0.1 g/cm^2 in the breast-fed group compared to 0.08 and 0.06 g/cm^2 in the formula-fed groups, 4 months 0.11 g/cm^2 in the breast-fed group compared to 0.08 and 0.06 g/cm^2 in the formula-fed groups. SEM appears to be about 0.02 for all data estimates.]** At 6 and 12 months, bone mineral content was the same in the formula-fed infants as in the historical control group of breast-fed infants. The authors posited that phytic acid (inositol hexaphosphoric acid) in soy formulas may interfere with calcium and zinc absorption, but that adaptation occurs and mineral absorption returns to normal over time. **[No comment was made concerning the effects of supplemental fluoride on measured bone mineral density. The expert panel did not believe there would be any effect of the supplemental fluoride at the doses provided, assuming recommendations at that time were adhered to.]**

Strengths/Weaknesses: Strengths include random assignment by infant sex, use of 2 soy formulas and a breast-fed comparison group, multiple measures of growth including head circumference, and use of blood measures and bone mineral content. Weaknesses include the small sample size (10 in each group) and the lack of adjustment for confounders. The separate historical control group used for breast-fed comparisons at 6 and 12 months was not optimal, and there could have been selection bias for breast-fed infants.

Utility (Adequacy) for CERHR Evaluation Process: This study is of limited utility in the evaluation process.

Cherry et al., 1968 *(511)*, supported by Gerber Products Co., tested the acceptance, tolerability, and nutritional value of a new (in 1968) soy formula provided to healthy newborn infants. When discharged from the hospital, mothers were given a 3-month supply of the soy formula or a marketed cow-milk formula (Modilac®). Formulas were packaged in identical containers. The soy formula contained isolated soy protein (15.1%), soy oil (37.5%), and carbohydrate (47.4% as sucrose and corn syrup solids). **[The Expert Panel notes that this soy formula contains more fat and less carbohydrate than soy formulas in current use. As noted in Chapter 1, current soy formulas include soy protein isolate (14.6–18%), vegetable oils (21–28.6%), and carbohydrate as corn syrup (40.8–55%) and sugar (10.2–11%).]** Formulas were approximately isocaloric. Mothers recorded the amount of formula consumed at each feeding, stool characteristics (color, consistency, and frequency), vomiting, acceptability of the feeding, and satiety after the feeding. Clinic visits included standard well-baby physical examinations, immunizations, measurements (weight, length, and head circumference), and blood work. Other procedures included muscle reflex patterns (twice in 6 months), x-rays of the tibia (at 6–8 weeks and again at 4–6 months), and more extensive blood work (at 2, 4, and 6 months). A reference population for growth measurements consisted of 200 children of similar socioeconomic and racial background chosen from the Collaborative Child Development Project at Charity Hospital in New Orleans. Fifty-eight of the 73 infants initially enrolled completed 6 months of follow-up. Proportions of infants with symptoms were compared by chi-squared testing, and growth parameters were compared using *t tests*. Multiple regression analysis was used to evaluate the relationship between nutritional components of the formulas and weight gain and ANCOVA was used to evaluate growth data and food intake.

Growth in soy formula-fed infants, especially girls, lagged behind cow milk-fed infants with significant differences primarily between 2 and 4 months of age. The reference population growth parameters (not shown) were generally between those of the cow-milk and soy groups. Formula intake level averaged 2 oz/day more in the cow-milk group than in the soy group, resulting in a 10% difference over the initial 56 days. The cow-milk group gained significantly more weight per oz of formula than the soy group. Cow milk-fed infants had greater intakes of methionine **[which today is added as a supplement to soy formulas],** calcium, and folic acid, and soy formula-fed infants had greater intakes of iodine, thiamine, vitamin C, and vitamin B12. Maternal observations showed more vomiting in the milk-fed group (21% slight, 5% frank) compared to the soy formula-fed group (13% slight, 1% frank, P=0.001). Eighteen percent of the infants in the milk-fed group were categorized as hungry after feeding compared to 7% in the soy formula-fed group (P=0.001). Loose stools were more frequently reported in the soy formula-fed group (27%) than the milk-fed group (19%, P=0.001). There were no instances of anemia, and hematologic parameters did not differ consistently by feeding group. The study authors concluded that the slower growth in soy formula-fed girls might be of concern.

Strengths/Weaknesses: Strengths including the blinding of participants to feeding-group assignment, measurements of head circumference and blood parameters, use of x-rays, an additional outside reference population, and assessment of differences in growth by infant sex. It is a strength that there was specific recording of the amounts of formula consumed at each feeding, allowing for recognition that the cow-milk group consumed more formula; however, there is no way to know if the accuracy of recording in the two groups differed. There could have been selection bias in mothers who elected not to breast feed and therefore could participate. The drop-out rate was high, with 15 of 73 subjects not completing the 6 months' evaluation. The sample was very small, providing limited power, especially for stratified analysis on infant sex. There was no comparison to exclusively breast-fed infants, and the introduction of solids was not specified. A repeated-measures type of analysis would have been appropriate for growth over time.

Utility (Adequacy) for CERHR Evaluation Process: This paper is limited utility for the evaluation process based on concerns about accuracy of intake recording and differences in modern soy formula from the product used at the time of the study.

Churella et al., 1994 ***(512)***, support not indicated, conducted a controlled, randomized, blinded, parallel clinical trial of 2 soy formulas in 64 healthy term infants. Infants were randomly assigned to Isomil® (protein 2.91 g/100 kcal) or a lower-protein (2.45 g/100 kcal) soy formula. Parents agreed to exclusively feed infants the assigned formula from approximately 2 to 112 days of age. Infants were evaluated at 8, 28, 56, and 112 days of age. Weight, length, and head circumference were measured at each evaluation. Blood samples were taken at 56 and 112 days and analyzed for plasma urea nitrogen, total protein, and albumin. Data were analyzed using ANOVA. No differences were detected between the groups in weight, length, or head circumference or in changes in these parameters. No differences in mean energy intakes calculated from 3-day formula intake records were detected. Mean protein intake was lower in the group fed the lower protein formula. Despite the significant differences in protein intake, no significant differences were detected between the 2 groups in total plasma protein concentrations, plasma albumin concentrations, or median plasma transthyretin concentrations. Plasma urea nitrogen concentration was lower in the low-protein formula group. The authors noted that the growth of the infants in their study was similar to that of infants in other studies who were fed human milk or cow-milk formula. They concluded that because transthyretin, a sensitive indicator of

protein status, was present at similar concentrations in both groups of infants, the content and quality of the protein in both formulas was adequate for growing infants.

Strengths/Weaknesses: Strengths include the controlled randomized design, the exclusive feeding for duration of trial, the multiple measures of growth including head circumference, use of blood measures, follow-up to about 3 months of age, and, relative to other studies, the moderate sample size (32 in each group). The power to show a group difference was limited by sample size, however, and the comparison was only between soy formulas without a formal comparison to other kinds of formulas or to breast feeding.

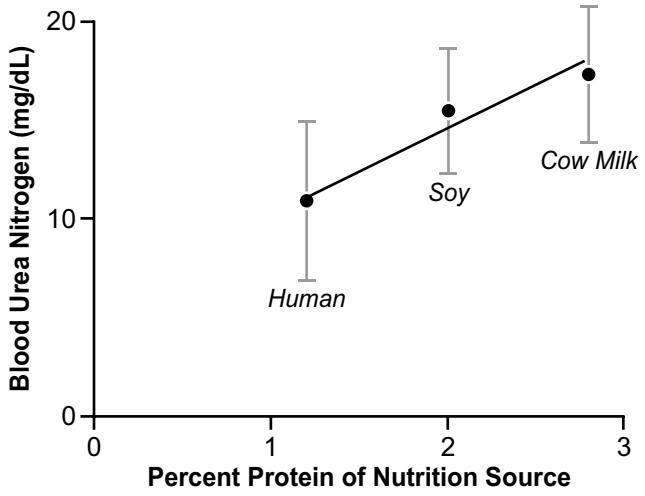
Utility (Adequacy) for CERHR Evaluation Process: This study is of no utility in the evaluation process.

Dean, 1973 ***(513)***, supported by Ross Laboratories, evaluated growth and development over the first 6 months of life in children fed a commercial soy formula or a commercial cow-milk formula, or who were breast-fed. Babies whose mothers chose to breast feed were enrolled into the breast-fed group. There were 26 such babies at the beginning of the study, but only 6 remained on breast feeding by 6 months of age. Among babies whose mothers chose to bottle-feed, 26 were assigned to soy formula (of whom 24 completed the 6-month trial) and 29 were assigned to cow-milk formula. There were 35 children in the cow-milk group at the end of the study because some breast-fed children who "failed" breast feeding within the first month of life were reassigned to the cow-milk formula group. **[The method of assignment to soy or cow-milk formula was not discussed.]** All babies were at least 2800 g at birth and were normal on examination by the investigator. Solid foods were added at 6–12 weeks of age. Weight, length, and head circumference were measured monthly. The Denver Developmental Screening test was administered **[how often and by who is not indicated]**. Blood was obtained at different visits in different children for chemistry and hematology evaluation.

The only abnormality noted in the children on soy formula was perianal redness that responded to zinc and cod-liver oil cream. The 24 children in this group who finished the study followed their centile growth curves during the study period. There was more disparity in growth in the cow-milk formula group, with 5 of 39 children having weight percentiles that exceeded their length percentiles. **[The number of children (39) in the study Results section is different from the number (35) in the study Methods section and from the number (40) in 1 of the study data tables.]** All infants were said to be "neurologically normal or advanced" except for 1 child in each of the 3 feeding groups. The laboratory values were normal in all children, but the blood urea nitrogen was highest in the cow-milk formula group, intermediate in the soy formula group, and lowest in the breast-fed group, proportional to the nitrogen content of the formulas/milk (**Figure 4**). The author concluded that soy formula was an adequate nutrition source.

Strengths/Weaknesses: Strengths include the use of 2 formula-fed groups and a breast-fed comparison group, repeated measurements of growth including head circumference, and developmental assessments. In spite of these strengths, the sample size was very small, and dropouts could have influenced the results. It seems inappropriate for infants who "failed" breast feeding to have been included in the cow-milk group. There could have been selection bias in mothers' assignment to feeding groups. The introduction of solid foods was not taken into account, and there was no report of how many calories were given to the groups. A repeated-measures analysis would have been useful.

Figure 4. Infant Blood Urea Nitrogen as a Function of the Protein Content of Food Source (Dean, 1973)



Human milk protein 1.2%, soy formula protein 2.0%, and cow-milk formula protein 2.8%. Data expressed as mean ± SD, from Dean, 1973 *(513)*.

Utility (Adequacy) for CERHR Evaluation Process: This study is of no utility for the evaluation process.

Fomon, 1959 *(514)*, supported by Mead Johnson and Ross Laboratories, evaluated growth and nitrogen retention in 4 term infants aged 4–6.5 months receiving a soy formula with 6.8% caloric protein. The author reported that weight gain was normal in these infants, and nitrogen retention was at least as great as that estimated in 3 of the infants during a previous month during which they consumed human milk.

Strengths/Weaknesses: This paper involved a small sample (n=4) with no comparison group. Nitrogen retention was compared to a time period 1 month earlier in a very small number of human milk fed infants. Exact intakes of soy formula were not provided.

Utility (Adequacy) for CERHR Evaluation Process: This paper has no utility in the CERHR evaluation process.

Hall et al., 1984 *(515)*, supported by Mead-Johnson Laboratories, conducted a randomized, controlled study to compare a calcium- and phosphorus-supplemented soy formula with a whey-predominant premature formula with regard to nutrition and growth in very low birth-weight infants. Forty infants from 3 to 8 weeks of age who had birth weights lower than 1500 g and were not breast-fed were recruited for the study. The mean (± SD) age at study entry was 16 (± 13) days in the soy formula group and 22 (± 14) days in the whey formula group. Formula assignment was made at the time of feeding initiation and all infants were given supplemental vitamins. Seventeen infants fed soy formula and 15 infants fed whey formula completed the study. Infants were put on cow-milk formula close to the time of hospital discharge. Body weight measurements were taken daily, and measurements of length and head circumference were taken weekly. Data were analyzed using Student *t* test.

No significant differences were detected in calorie or protein intake or in formula tolerance between groups. At 8 weeks of age, the mean body weight of infants receiving whey formula was significantly higher than that of infants receiving soy formula. There was a significantly higher mean weight gain per day in the whey formula group during the last 2 weeks of the study, in addition to greater caloric efficiency (grams weight gain/100 kcal/kg/day) at 7 weeks in this group. Similarly, mean weight gain over the duration of the study was significantly greater in the whey formula group. Mean serum protein and albumin concentrations were within the normal range for both groups, although values were significantly higher in the whey group at the end of the study. Mean serum calcium and phosphorus were significantly higher at 8 weeks in the whey formula group, although the values for both groups were within normal ranges. Concentrations of alkaline phosphatase, parathyroid hormone, and 25-hydroxyvitamin D were within normal ranges and no differences were detected between groups. No cases of rickets were identified in either group by wrist radiography.

The study authors concluded that premature whey formula was preferable to supplemented soy formula for very low birth-weight infants.

Strengths/Weaknesses: The prospective design, use of a homogeneous, special population sample of very low birth-weight infants, and the repeated measurements for growth including head circumference are strengths. Weaknesses include the small sample size and the initiation of subjects into the study at 3 weeks of age or older without adjustment for baseline differences.

Utility (Adequacy) for CERHR Evaluation Process: This study has no utility in the evaluation process.

Hillman et al., 1988 [516; 517], supported by Mead Johnson Co., studied 31 term infants receiving either human milk (n=9), the cow-milk formula Enfamil® (n=11), or the soy formula ProSobee® (n=11). The decision to breast- or formula-feed was made by each infant's mother. Within the formula group, babies were placed on soy formula by their private pediatricians for "colic or spitting up." Cereal was allowed at 4 months of age, and other solid foods at 6 months. Breast-fed infants were given vitamin D 400 IU/day as a supplement (both formulas also included this level of vitamin D). The infants were examined at entry into the study (age <3 weeks) and then at 2, 4, 6, 9, and 12 months. Samples were taken for measurement of serum vitamin D, 25-hydroxyvitamin D, 1,25-dihydroxyvitamin D, parathyroid hormone, alkaline phosphatase, calcium, phosphorus, magnesium, and albumin. One urine sample was also taken randomly from each subject for measurement of calcium, phosphorus, magnesium, and creatine. Weight, body length, head circumference, bone width, and bone density (single-photon absorptiometry, mid-humerus) were measured at each visit. Trends in data over time were analyzed by linear regression, and mean slopes were analyzed using the 2-tailed Dunnett *t* test. ANOVA was performed for 6-month values, and differences were identified by post hoc Duncan test.

No significant differences by feeding type were detected in the rate of increase of bone width or bone mineral content. Serum and urine measurements are shown in **Table 84**. The differences noted in vitamin and mineral levels were considered to reflect homeostatic adjustments to differing mineral levels in the food sources. The authors concluded that infant bone mineralization was not affected by food type when adequate vitamin D and mineral intake were ensured.

Strengths/Weaknesses: The use of 3 feeding groups is a strength, as are the multiple measurements of growth, including measurements of head circumference and bone density and the blood and urine

Table 84. Laboratory Values at 6 Months of Age (Hillman et al., 1988)

Analyte	*Human milk (n = 8)*	*Enfamil (n = 11)*	*ProSobee (n = 11)*
Serum Measurement			
Vitamin D (ng/mL)	2.2 ± 0.9	2.0 ± 0.8	2.3 ± 1.2
25-Hydroxyvitamin D (ng/mL)	25.6 ± 3.3	23.8 ± 3.1	24.0 ± 5.3
1,25-Dihydroxyvitamin D (pg/mL)	29.3 ± 11.3	45.4 ± 15.9[a]	47.7 ± 10.2[a]
Calcium (mg/dL)	10.0 ± 0.6	9.9 ± 0.6[b]	10.5 ± 0.5 a,[b]
Phosphorus (mg/dL)	5.5 ± 0.3	6.0 ± 0.7[a]	6.6 ± 0.9[a]
Magnesium (mEq/L)	1.81 ± 0.13	1.79 ± 0.13[b]	1.95 ± 0.15 a,[b]
Alkaline phosphatase (IU/L)	78 ± 22	59 ± 8	70 ± 23
Parathyroid hormone (µlEq/mL[c])	3.2 ± 2.5	2.2 ± 0.4	3.1 ± 0.8
Albumin (g/dL)	4.7 ± 0.4	4.5 ± 0.3	4.5 ± 0.6
Urine Measurement			
Calcium (mg/mg creatinine)	0.37 ± 0.31	0.25 ± 0.14	0.29 ± 0.28
Phosphorus (mg/mg creatinine)	0.71 ± 0.44	1.4 ± 0.3[a]	1.5 ± 0.66[a]
Magnesium (mEq/mg creatinine)	0.018 ± 0.008	0.018 ± 0.005[b]	0.029 ± 0.017[a,b]

Values are mean ± SD.
[a] Different from human milk: $P < 0.05$.
[b] Difference between ProSobee® and Enfamil®, $P < 0.05$.
[c] Units as in the original.
From Hillman et al., 1988 *(517)*.

measures. The specification of times when solid foods were allowed is a strength, but it is a weakness that solid food was started early and there was no documentation of the dietary intakes of the 3 groups. Other weaknesses include the small sample size and the lack of adjustment for potential confounders. In addition, the soy group was selected for a specific indication, and these children could have been on cow-milk formula before enrollment (up to 3 weeks). Using "colic" as an indication for recommending soy infant formula introduces a potential bias in group assignment. Since colic or spitting up was used as an indication, the timing of introduction of the soy-based formula needs to be stated even though the outcomes were not different.

Utility (Adequacy) for CERHR Evaluation Process: This study is of limited utility for the evaluation process, except for the bone mineral density portion of the study, which may be of high utility despite very small sample size.

Jung and Carr, 1977 ***(518)***, support not indicated, studied the nutritional and growth differences between 20 infants on soy formula (Isomil®) and 20 infants on cow-milk formula (Similac® with Iron). Healthy infants were assigned by gestational age, weight, race, and sex to receive 1 of the formulas for 16 weeks. Cereal was introduced at 4 weeks, and vegetables were introduced at 8 weeks. Infants were assessed for formula tolerance, weight, length, and head circumference. The researchers reported that general acceptance of both formulas was good, and no infants needed formula changes. All growth measurements were normal, and no significant differences in body weight, length, or head circumference were observed between the groups. Hemoglobin, hematocrit, protein, albumin,

calcium, phosphorous, glucose, urea nitrogen, and alkaline phosphorous were also within the normal range and did not significantly differ between groups. The authors conclude that the soy formula used in this study imposed no significant nutritional differences compared to cow-milk formula.

Strengths/Weaknesses: There was some attention to prospective matching of infants on important confounders. The introduction of specified solids at specified times in both groups is a strength, as is the use of blood measures and measurement of head circumference. The sample size was small, however, with limited power to detect differences between groups. There was inadequate adjustment for potential confounders, and there was no breast-fed comparison group. Time of introduction of feedings could be more clearly stated.

Utility (Adequacy) for CERHR Evaluation Process: This study is of limited utility for the evaluation process.

Kay et al., 1960 ***(519)***, supported by the Borden Company, followed normal, healthy infants fed soy formula (Mull-Soy®) or evaporated cow-milk formula (Silver Cow®) to evaluate differences in weight, length, hemoglobin, hematocrit, and plasma biochemical values. **[An additional 32 infants on unspecified feeding methods were evaluated at birth or at 3 months of age and are not considered here**.] At 24 hours of age, 14 infants were started on soy formula and 14 were started on evaporated cow-milk formula and followed for 3 months. Efforts were made to match patients for sex, race, and birth weight. Infant weight and length were measured at clinical visits every 2 weeks. Hemoglobin, hematocrit, and plasma biochemical values were measured from blood drawn shortly after birth and at 3 months of age. Plasma protein electrophoresis was performed to quantify albumin, α1-globulin, α2-globulin, β-globulin, and γ-globulin.

Growth increases in the soy formula and evaporated milk formula were similar and paralleled normal growth curves (based on Iowa Growth Charts). No statistically significant feeding group difference were identified in hemoglobin, hematocrit, or plasma biochemistry values except as shown in **Table 85**. The study authors concluded that nutritive value and infant growth measurements were similar in infants fed soy formula and evaporated cow-milk formula from birth to 3 months of age.

Table 85. Plasma Biochemical Values at 3 Months of Age in Children Given Soy Formula or Evaporated Cow-Milk Formula (Kay et al., 1960)

Laboratory Test	*Group*	*n*	*Mean ± SD*	*P-value by t-test*
Cholesterol, mg. %	Soy	14	126 ± 34	0.01
	Cow milk	14	163 ± 32	
Total plasma protein, g %	Soy	14	6.7 ± 0.8	0.03
	Cow milk	14	6.0 ± 0.5	
α2-Globulin	Soy	7	8 ± 4	0.01
	Cow milk	8	14 ± 5	
γ-Globulin, %	Soy	7	9 ± 3	0.001
	Cow milk	8	15 ± 4	

From Kay et al., 1960 *(519)*.

Strengths/Weaknesses: Some effort was made to match groups at the outset on important potential confounders. The use of blood measures at two time points is a strength. Although there was a control group, diet was unrestricted and there was no comparison to breast-fed infants. It was unclear if infants were exclusively formula-fed for the duration of study and how they were selected for feeding groups. The small sample size provided limited power for the conclusion of no differences in growth curves, and there was no measurement of head circumference. Only about half of the already small sample size was available for the full set of biochemical measures at 3 months of age. Intake was not reported, and it is like that the evaporated milk group would have had down-regulation of intake. Without knowing whether feedings were isocaloric, the groups cannot be meaningfully compared. The differences in total protein α2-globulin levels are small and not clinically significant.

Utility (Adequacy) for CERHR Evaluation Process: This paper has no utility in the evaluation process given that the feedings are not comparable and evaporated cow's milk is not relevant to the present and only of historic value. Protein content of the feeds would also differ vastly.

Köhler et al., 1984 ***(520)*****,** support not indicated, reported the growth and food intake of 59 infants aged 6–25 weeks on human milk, cow-milk formula, or soy formula. Participants were selected by 6 weeks of age from 2 well-baby clinics, 1 of which provided infants with cow milk-based formula (Similac®; n=20), and the other of which provided infants on soy formula (Isomil®; n=13). The remaining children (n=26) were fully breast-fed. Mothers recorded 48-hour intake at 6, 14, 22, and 26 weeks. Cow-milk and soy formula consumption was measured by administering a standard formula volume and weighing the amount remaining after feeding. Breast-fed infants were weighed by their mothers before and after feedings. There were no significant differences in consumption between formula groups, but breast-fed infants consumed significantly less per 24 hours than the formula-fed infants. There were no statistically significant differences in energy intake between infants on soy and cow-milk formulas. The difference in intake between breast-fed and all artificially fed infants was statistically significant at 6 and 14 weeks **[statistical methods not indicated].**

Growth was measured by weight, length, and skin-fold thickness and followed for 52 weeks. Infants on soy formula gained less weight in the first 6 weeks than breast-fed infants and those on cow-milk formula. **[Infants were selected by six weeks of age; therefore, time 0 should have been considered the day they were assigned with follow-up adjustment for the confounding variable of birthweight.]** The authors suggested that this difference might be explained by infants in the soy group being significantly heavier by an average of 200 g at birth. After 6 weeks of age, no significant differences in mean weight were detected between the 3 groups. No significant differences were detected between the 3 groups in length or sum of skin-fold thicknesses.

Radiographs of the left hand were taken at 3 and 6 months of age to measure bone mineralization and maturation. The internal (d) and external (D) diameters of the narrowest part of the diaphysis of the 2nd metacarpal bone were used to "represent" the cortical cross-sectional area as D^2-d^2. **[The Expert Panel notes that the cross-sectional areas would be $\pi(D^2-d^2)/4$.]** This measurement, which the authors cite as representative of bone ash content per unit length, was used to indicate bone mineralization. Ossification centers were measured to characterize skeletal maturation. At 3 months of age, the soy formula group showed slower mineralization and bone maturation than both the breast-fed group and the cow-milk group; however, by 6 months these differences in skeletal development were no longer statistically significant.

The authors concluded that although they believed birth weight differences could explain differences in weight gain during the first 6 weeks of life, they could not exclude the possibility that nutrients were less well absorbed from soy formula than from human or cow milk.

Strengths/Weaknesses: It is a strength that this study compared three feeding groups with recording of infant intake and measures of bone mineralization. Multivariate analysis should have been used to adjust for differences such as birth weight between groups. There could have been selection bias among mothers who elect to breast feed or not. Assignment to formula feeding groups was by clinic location and not within clinics, and there could have been differences between women associated with a site that were not taken into account. There was 1-year growth follow-up, but no head circumference measures were taken, and there was no mention of adjusting for supplementary foods, including milk in the breast-fed group. The sample size was small with inadequate adjustment for other potential confounders.

Utility (Adequacy) for CERHR Evaluation Process: This study is of limited utility for the evaluation process.

Kulkarni et al., 1980 *(521)*, funding not indicated, reported 22 cases of rickets in very low birth-weight (< 1500 g) infants fed a soy-isolate formula. The soy formula contained 700 mg/L calcium, 500 mg/L phosphorus, and 400 IU/L vitamin D. When feeding was tolerated, the formula was supplemented by a multivitamin; additional calcium lactate and/or vitamin D were also provided depending on neonatologist preference. The mean daily intake of each of these supplements was measured during infant hospitalization periods. The lowest mean serum phosphorus concentration occurred at 7–10 weeks, which preceded the active phase of rickets by about 2 weeks. The highest serum levels of alkaline phosphatase occurred at 11–14 weeks, 2 weeks prior to the healing phase. The lack of a control group prevented the authors from drawing conclusions from the data, and no statistical analyses were performed. However, the authors suggested further studies investigating calcium, phosphorus, and vitamin D in very low birth-weight infants with rickets, in particular those fed soy formula. This report led to a controlled prospective study, Kulkarni *et al.*, 1984 *(522)*, which is discussed below.

Strengths/Weaknesses: This case series involved a special population of very low birth-weight infants.

Utility (Adequacy) for CERHR Evaluation Process: Although this report may be useful for hypothesis-generation, it has no utility in the CERHR evaluation process.

Kulkarni et al., 1984 *(522)*, supported by Ross Laboratories, performed a clinical trial in 46 very low birth-weight (≤1500 g) infants given 1 of 3 formulas to ascertain the relationship between formula type and rickets. Infants were randomly assigned to soy formula (Isomil®), cow-milk formula (Similac® with Iron), or a hypercaloric cow milk-based formula designed for low birth-weight infants (Similac® 24 LBW), and followed until 3–4 months of age. Formula compositions are given in **Table 86**.

Formula was introduced by continuous nasogastric drip until a weight of 1600 g was reached and infants could tolerate bolus feeding. Roentgenograms of 1 knee and 1 wrist were taken in all infants when they were between 8 and 17 weeks old. Infants who did not reach a daily alimentary intake of 80 kcal/kg by 5 weeks old were not included in the study. Initial numbers of infants in the soy formula, cow milk-based formula, and hypercaloric cow milk-based formula groups were 15, 18, and 13, respectively; however, these numbers dropped to 9, 8, and 8.

Table 86. Nutritional Composition of the Formulas (Kulkarni et al., 1984)

Component	*Soy*	*Cow Milk*	*Hypercaloric Cow Milk*
Energy, kcal/L	680	680	810
Protein, g/L	20.0	15.5	22.0
Fat, g/L	36.0	36.1	44.9
Calcium, mg/L	700	510	730
Phosphorous, mg/L	500	390	560
Vitamin D, IU/L	400	400	480

From Kulkarni et al., 1984 *(522)*.

Nutrient intake was analyzed by repeated measures ANOVA and the Scheffé method of multiple comparisons. No significant formula-group differences were detected in intake of calories, protein, phosphorus, or vitamin D. Calcium intake was significantly higher in the soy formula-fed infants than those fed cow milk-based formula. Blood chemistry data collected during hospitalization were analyzed using the Bonferroni multiple comparison test. Data collected after discharge were averaged for each infant, and the values were analyzed using ANOVA followed by the Duncan multiple range test.

No significant differences were found prior to 3 weeks of age. Infants on soy formula had significantly lower serum levels of phosphorus at weeks 3, 5, and 8–14 and higher serum levels of alkaline phosphatase at week 5 and weeks 8–14 than infants in 1 or both of the cow-milk formula groups. Active rickets was diagnosed by x-ray based on the concurrence of 2 radiologists. Of the 15 infants in the soy formula group, 9 developed rickets. In contrast, only 1 case of rickets was diagnosed in the 2 cow-milk groups combined. The study authors speculated that the higher incidence of rickets in very low birth-weight infants fed soy formula may have been due to decreased absorption or solubility and bioavailability of minerals and discouraged the use of soy isolate formula for this population.

Strengths/Weaknesses: Strengths include the study of a homogeneous special population of very low birth-weight infants, random assignment to 1 of 3 formula groups, similarity of caloric, protein, phosphorus, and vitamin D intake between groups, follow-up to 3–4 months, specification of the mode of delivering formula, specification of exclusionary criteria, use of appropriate repeated-measures method of analysis, and the use of 2 radiologists to confirm the diagnosis of rickets. **[Caloric similarity would only have occurred if volume was restricted to a certain caloric intake, otherwise, protein to calorie ratios would vastly differ between the groups.]** Weaknesses include the small sample size and high drop-out rate and variation in feeding.

Utility (Adequacy) for CERHR Evaluation Process: This study is of limited utility for the evaluation process to assess the risk for rickets in very low birth-weight infants.

Lasekan et al., 1999 ***(523)***, supported by Ross Products Division, Abbott Laboratories, performed a randomized, masked, parallel 1-year clinical feeding study to determine the effects of soy protein-based formulas and supplemental free nucleotides on the growth of infants. Healthy term infants were recruited from 9 private pediatric practices. Infants were given a soy formula (Isomil®, n=73), the soy formula with 72 mg/L supplemental free monomeric nucleotides (n=73), or mixed feeding (n=67) consisting of 2 months exclusive breast feeding followed by a cow-milk formula (Similac® with Iron supplementation),

without nucleotide fortification. Assignment was randomized by sex in the 2 soy groups. Infants were enrolled in the mixed-feeding group if their mothers chose to breast feed. Children were allowed to eat solid foods after 4 months of age. Formula tolerance and growth measurements (weight, length, and head circumference) were measured at enrollment and at 0.5, 1, 2, 4, 6, 7, and 12 months of age. Growth data were analyzed by 2-way ANOVA with feeding and sex as interaction terms.

At birth, infants on soy formula were heavier and longer than infants on nucleotide-supplemented soy formula (mean ± SEM weight: soy 3616 ± 66 compared to soy + nucleotides 3254 ± 78 g; length: soy 52 ± 0.4 compared to soy + nucleotides 50 ± 0.4 cm, $P<0.05$). There was a significantly larger weight gain between 2 weeks and 1 month of age in the mixed-feeding group (mean ± SEM: 42.6 ± 1.8 g/day) compared to infants fed unsupplemented soy formula (34.1 ± 1.3 g/day) or soy formula with supplemental nucleotides (36.7 ± 1.3 g/day). Between 2 and 4 months of age however, infants fed unsupplemented (24.2 ± 0.7 g/day) and supplemented soy formula (24.2 ± 0.8 g/day) gained significantly more weight than mixed-feeding infants (20.3 ± 0.3 g/day). After 4 months of age, no significant weight gain differences were detected between the groups. Children in the unsupplemented soy group were significantly longer than mixed feeding infants at 7 and 12 months of age. Mean head circumference for infants on soy formula with supplemental nucleotides was larger than the mixed-feeding group at 6 months of age. Over the course of the entire study, no differences in length and head circumference measurements between groups were detected. All growth measurements were consistent with National Center for Health Statistics normal values.

Mean hemoglobin concentration, plasma albumin, and blood urea nitrogen were within the normal ranges in all groups. There was a significantly higher level of blood urea nitrogen in both soy groups than in the mixed-feeding group ($P<0.0001$, mean ± SEM not reported). The authors noted that this finding was expected due to the typically higher levels of protein in soy formula than in human milk.

The study authors concluded that over the first year of life, growth of healthy term infants fed soy formula or soy formula with supplemental nucleotides was similar to infants on a mixed-feeding regimen of human milk followed by cow milk-based formula. They also noted that they could detect no significant effects of supplemental nucleotides on growth.

Strengths/Weaknesses: Strengths include the use of 3 defined feeding groups, the relatively large sample size, random assignment by sex to soy-feeding groups, the specified time for introduction of solid foods, and the multiple measures of growth including head circumference. The mixed-feeding was group defined by length of exclusive breast feeding followed by cow-milk formula. There may have been selection bias in mothers who elect to breast feed. Random assignment by sex to the two formula groups did not result in equality between the groups; with 73 children in each group, there was still a mean difference of almost 400 g in birth weight between the groups, suggesting that there was some other fundamental difference between the groups at baseline. There was inadequate adjustment for potential confounders, and a repeated-measures analysis would have been preferable.

Utility (Adequacy) for CERHR Evaluation Process: This study is of limited utility for the evaluation process.

Mimouni et al., 1993 ***(524)***, supported by the NIH, Children's Hospital Research Foundation, and Ross Laboratories, studied 72 healthy term infants receiving human milk (n=10), cow-milk formula

(Similac®, n=20), or 1 of 2 soy formulas (Prosobee®, n=21 or Isomil® n=21). Breast-fed infants were recruited from infants whose mothers had decided to breast feed and were given vitamin D 400 IU. Supplementation of feeds with cow-milk formula was permitted. Infants whose mothers had decided not to breast feed were randomly assigned to a formula group. Solid food was withheld until 3 months for breast-fed infants and until 4 months for formula-fed infants. The infants were examined at entry into the study (2–7 days of age) and then at 8, 16, 26, and 52 weeks for measurement of weight, length, head circumference, bone mineral content (single-photon absorptiometry, distal radius and ulna), and serum calcium, phosphorus, magnesium, alkaline phosphatase, and 1,25-dihydroxyvitamin D. Data were analyzed using chi-squared for discrete variables and ANOVA or nonparametric methods for continuous variables. The Tukey studentized range test was used to identify pair-wise differences.

No differences in weight and head circumference by feeding group were detected, but length gain was significantly lower in the breast-fed group than in the 3 formula-fed groups from 1 to 26 weeks. Bone mineral content and bone width were similar between groups throughout the study. No significant feeding group effects on levels of serum total or ionized calcium, magnesium, or alkaline phosphatase were detected. Serum phosphorus was significantly lower in the breast-fed group than in the combined formula groups at 8 weeks and lower than in the cow-milk formula group at 26 weeks. Serum levels of 1,25-dihydroxyvitamin D were higher in the Prosobee® group than in the other groups at 8, 16, and 26 weeks.

The authors suggested that elevated concentrations of 1,25-dihydroxyvitamin D in Prosobee®-fed infants could indicate inadequate mineral intake or high mineral need. They indicated, however, that the increase in 1,25-dihydroxyvitamin D was apparently sufficient to increase calcium absorption and resulted in adequate bone mineral content. The authors concluded that the concentration and suspension characteristics of minerals in available soy-based formulas permitted adequate growth and bone mineralization.

Strengths/Weaknesses: Strengths include the use of 4 feeding groups, including 2 types of soy formula, and random assignment to formula-fed groups. The 1-year follow-up and multiple measurements of growth including head circumference and bone mineral content are additional strengths. There could have been selection bias for breast-fed infants, however, and supplementation with cow-milk formula was allowed for breast-fed infants. Solid foods were restricted for differing lengths of time in breast-fed and formula-fed infants and were introduced early. The sample size was relatively small.

Utility (Adequacy) for CERHR Evaluation Process: This study is of limited utility in the evaluation process.

Naude et al., 1979 ***(525),*** support not indicated, conducted a 35-day trial in 40 preterm infants to compare the effects of feeding a cow-milk formula compared to a soy formula. Infants who weighed between 1500 and 1800 g at birth, appeared healthy, were appropriate for gestational age, and whose mothers had decided not to breast feed were randomly assigned to either a cow-milk or a soy formula feeding group. The children received formula amounts up to 180 mL/kg bw/day. All infants were given supplemental vitamins. Weights were taken on study days 1 and 2 and then on alternate days for the duration of the study. Head circumference and body length were measured on day 1 and then weekly. Blood samples were taken once between days 7 and 10 and again between days 32 and 35. Samples were analyzed for serum concentration of albumin, calcium, phosphorus, alkaline phosphatase, and sodium, and for blood urea nitrogen and hemoglobin. Student *t* test was used for statistical analysis.

The researchers report that it became immediately evident that the infants in the soy formula group were not progressing well. Of the first 10 infants allocated to this group, 7 were withdrawn because of weight loss or poor weight gain, and 1 died with necrotizing enterocolitis. Only 2 infants completed the 35-day trial. Only 2 of the first 10 infants in the cow-milk group were withdrawn, 1 due to jaundice and the other due to vomiting and a diaphragmatic hernia. The remaining 8 were said to progress "satisfactorily." Due to the low success rate, the researchers changed the feeding regimen for the 20 subsequent subjects, increasing the daily intake from 180 to 200 mL/kg bw/day. The remaining infants allocated to the soy-formula group progressed more satisfactorily with the increased intake, and only 2 were withdrawn due to jaundice. On the higher-volume feeds, infants on soy formula gained an average of 305 g less weight and acquired 1.6 cm less height than infants on cow-milk formula. **[The data table shows a length difference of 16 cm, which the Expert Panel assumes should be 16 mm.]** Serum albumin and phosphorus levels were lower in soy formula-fed infants. The study authors recommended that caution be exercised in the feeding of soy formulas to preterm babies.

Strengths/Weaknesses: Strengths include the random assignment to soy or cow-milk formula, the multiple growth measurements including head circumference, the use of blood measures, and the use of a homogeneous special population sample of preterm, low birth-weight infants. Weaknesses include the small sample size and inability to adjust for potential confounders. In addition, the change in strategy mid-stream made it unclear whether reduced growth in subsequently enrolled soy formula-fed infants was due to a still-inadequate supply of formula.

Utility (Adequacy) for CERHR Evaluation Process: This study has no utility in the evaluation process.

Sellars et al., 1971 ***(526)***, supported by Borden, Inc., reported length and weight measurements for 1583 Caucasian infants during the first year of life. Mothers were encouraged to breast feed as long as possible. Infants not being breast-fed, or being weaned from breast feeding, were assigned to cow-milk or soy formula based on family history of allergy **[although the assignment strategy was not discussed]**. The comparison groups for evaluation of length and weight included 401 breast-fed infants, 239 infants on soy formula, and 839 infants on cow-milk formula. Infants were evaluated monthly until the age of 6 months and bi-monthly thereafter. Length and weight were plotted on growth curves, and statistical comparisons were made by an unspecified method. No differences in growth were detected between the 3 feeding groups. The authors concluded that any of the 3 feeding methods would support normal growth.

Strengths/Weaknesses: The large sample, repeated measurements of weight and lengths, and 1-year follow up are strengths. The study was limited to 1 racial group, and there may have been selection bias in mothers who did not breast feed or who discontinued breast feeding, as well as in assignment to soy formula group on the basis of family history of allergy. Head circumference was not evaluated. Growth curves should have differed between breast-fed and formula-fed infants at least before introduction of solid foods; so, it the lack of detail on the method of statistical comparisons is especially problematic. There were apparently no adjustments for potential confounders and baseline differences between groups. The introduction of solid food by pediatricians at 1 month is another weakness. Timing of weaning to the different feedings was not provided.

Utility (Adequacy) for CERHR Evaluation Process: This paper is of limited utility in the evaluation process.

Seppo et al., 2005 ***(527)*****,** funding support provided by Turku University Hospital, the Social Insurance Institute, and the University Hospital for Skin and Allergic Diseases in Finland, conducted a study to compare growth and nutritional indices in male and female infants with cow's milk allergy fed either a soy formula or a extensively hydrolyzed whey formula (HWF). Infants with confirmed cow's milk allergy were randomly assigned to receive either soy formula (n=84, mean starting age=7.8 months) or extensively hydrolyzed whey formula (n=84, mean starting age=7.5 months). Blood samples were collected at the start of the study and when infants were 1 and 2 years of age. A two-day dietary recall was completed by the parents when the infants were 1 and 2 years of age. Length and weight measurements were made at 0, 6, 8, 10, 12, 18, 24, 36, and 48 months of age. Length (measured in SD score) and weight (measured in weight-for-length, expressed as the percentage deviation from the median weight-for-length according to sex) were based on Finnish reference growth data. Repeated measures ANOVA was used to analyze nutrient intake, laboratory results of blood analyses, length, and weight.

A number of infants switched formula by the age of 2 due to suspected adverse reaction to the formula (21/84 or 25% in the soy group and 5/84 or 6% in the HWF group). Adverse reaction to formula was confirmed in 8 of 84 (9%) infants in the soy formula group and 2 of 84 (2%) infants in the HWF group. At the age of 2 years, 70% of infants were still being fed the study formulas. Infants in the soy formula group received a higher percentage of energy from formula compared to the HWF group. Dietary intakes of zinc and vitamin E were significantly lower in the HWF infants than in the soy formula infants, and the intake of riboflavin was significantly higher in the HWF than in the soy formula group. There were no significant differences between the groups in percentages of abnormally low laboratory values (mean cell volume, hemoglobin, zinc, and ferritin) or in the percentage of high alkaline phosphatase. Both groups had smaller weight-for-age growth measures compared to Finnish reference data, a finding consistent with other reports of reduced growth in infants with cow's milk allergy, however growth measured did not differ between the formula groups based on data collected during the period of 1 to 4 years old. The authors concluded that nutritional status and growth did not differ between the groups and the selection of formula can be made on the basis of tolerance and, to some extent, cost.

Strengths/Weaknesses: The infants were offered soy based on cow milk allergy. Since there is an approximately 14% cross reactivity with soy if an infant is allergic to cow milk protein, knowledge of the adverse events is important. This study reported that 9% and 2% of infants had confirmed adverse effects to soy or hydrolysate formula, a finding that does not add much to the consideration of soy toxicity. Also, it is unclear whether milk was the major source of nutrition after the weaning period or whether complementary foods were introduced.

Utility (Adequacy) for CERHR Evaluation Process: This study is of limited utility for the evaluation.

D'Auria et al., 2006 ***(528)*** commented on the study conducted by Seppo *et al.*, 2005 *(527)* stating that the study would have been strengthened by including indices of protein metabolism, i.e., total serum protein, albumin, prealbumin, and urea nitrogen, to address the hypothesis that hydrolyzed formulas supply excessive amounts of amino acids. **[The expert panel notes that this is not true; hydrolysis results in smaller dalton size of the protein.]** D'Auria suggested also suggested that soy formula not be used in infants with cow's milk allergy who are less than 6 months of age; the percentage of infants with an adverse reaction to the formula was higher in the soy group and there are reports of more frequent adverse reactions to soy in younger infants compared to older infants.

Shenai et al., 1981 ***(529)*****,** supported by Ross Laboratories, prospectively studied nutritional retention over 28 days in 19 very low birth-weight (<1530 g) infants on either soy formula (similar to Isomil®; n=10) or cow milk-based formula (Similac® with Iron; n=9). Infants with a gestational age of 31 weeks or less were recruited from the neonatal intensive care center of the University of Oregon Health Science Center. The very low birth-weight infants were of normal size for gestational age and did not have metabolic or congenital anomalies. Within 7 days of birth, infants were fed at 3-hour intervals with the assigned formula in addition to a multivitamin and vitamin E 25 IU/day. When the infant had achieved full oral intake of the formula for 8 days, a 96-hour metabolic balance study was performed. Infants were weighed daily and occipitofrontal circumference and crown-heel length were measured weekly. Stool and urine samples were frozen until analysis for calcium, phosphorus, and nitrogen; stools were also analyzed for fat. Blood samples were taken at approximately 4, 14, 21, and 28 days postnatally. The 2 groups were studied successively **[not randomized]**; however, stool and urine analyses were concurrent. Statistical analysis was performed by *t* test and ANCOVA.

No formula-group differences in fat intake or absorption were detected. The soy formula-group had a significantly higher intake of nitrogen and calcium and lower intake of phosphorus than the cow-milk group. Because there were no detected significant differences in calcium excretion, absorption, or retention rates, the authors proposed that the absence of lactose in soy formula did not interfere with calcium retention or homeostasis. Though nitrogen intake was higher in the soy formula- than cow-milk formula-group, there was significantly lower nitrogen retention in the soy-formula group. Soy formula-fed infants had significantly lower phosphorus intake, absorption, and urinary excretion and a higher fecal excretion than cow-milk formula-fed infants. However, no significant difference in overall phosphorus retention between groups was detected.

No significant differences were detected between groups at any time in serum calcium or alkaline phosphate concentrations. Mean serum phosphorus levels were lower in soy formula-fed than cow milk-fed infants on PND 14, 21, and 28. No significant differences were found in mean serum total protein and albumin values at any time, and measurements were within the normal range for very low birth-weight infants. Mean blood urea nitrogen values were significantly higher in the soy-formula group on PND 14, 21, and 28 compared to the cow-milk group. Weight gain, crown-heel length, and occipitofrontal circumference did not significantly differ between the 2 feeding groups.

The authors stated that the study period was too short for these measurements to be valuable in determining growth effects. The authors also noted that the study may not have shown effects of sustained soy-formula feeding due to the small sample size, short duration, and lack of long-term follow-up. They specifically questioned whether long-term use of soy formula in very low birth-weight infants would have led to phosphorous-deficiency rickets.

Strengths/Weaknesses: The use of a homogeneous, special population sample of very low birth-weight infants and the repeated measurements for growth including head circumference and blood samples are strengths; however, the follow-up period of less than 1 month was too short, and the small sample size gave very limited power to detect differences.

Utility (Adequacy) for CERHR Evaluation Process: This study has no utility in the evaluation process.

Steichen and Tsang, 1987 ***(530)*****,** supported by the National Institute of Child Health and Human Development (NICHD), the Children's Hospital Research Foundation, the Veteran's Administration of Cincinnati, the Jewish Hospital of Cincinnati, Ross Laboratories, and Gerber Laboratories, performed a prospective, randomized, double-blind, longitudinal study over 1 year to determine differences in growth and skeletal mineralization between infants fed soy formula and those fed cow-milk formula. Subjects were healthy term infants appropriate for gestational age and born to healthy, middle-class white mothers. Infants were given a soy formula (Isomil® with Iron, n=18) or a cow-milk formula (Similac® 20 with Iron, n=17). Infants were fed exclusively with the assigned formula from birth until 6 months of age, when baby food was provided by investigators and introduced to the diet. At 6 weeks and 3, 6, 9, and 12 months, measurements were made of weight, length, head circumference, and bone mineral content by dual photon absorptiometry of the midshaft and wrist of the left radius and ulna. Student *t* test and ANOVA with Duncan multiple range test were used in the comparisons.

There were no significant differences at the start of the study in weight, length, or head circumference. Energy intake during the study was the same in both groups, and no significant differences were detected between the groups in weight, length, head circumference, or the rates at which these measurements increased over the study period. All infants were within the normal growth range based on the National Center for Health Statistics growth curves. The ratio of bone mineral content to bone width was significantly lower in the soy formula group at 3, 6, 9, and 12 months of age. Both groups showed a decrease in bone mineral content per bone width until 26 weeks of age and then a subsequent increase until 52 weeks. In the infants on cow-milk formula, bone mineral content rebounded to initial levels, but in the soy formula-fed infants, bone mineral content remained significantly lower than initial values.

The authors noted that the lower bone mineral content of the soy formula-fed infants may not have been clinically significant and was similar to that of breast-fed infants measured at 3, 6, and 12 months of age in a previous study at the same center. They further suggested that cow-milk formula may cause bone to be hypermineralized, rather than soy formula causing below-normal bone mineralization. Although the authors did not analyze whether there was benefit or harm due to increased bone mineral content, they noted that in rats, bone hypermineralization early in life was found to decrease long-term mineral retention, and they questioned a relationship to osteoporosis. The authors also speculated that the lower bone mineral content in soy formula-fed infants could have been due to decreased availability of calcium and protein.

Strengths/Weaknesses: The prospective, randomized, double-blind design is a strength. There was 1-year follow-up with multiple measures, including head circumference and bone mineral content. Use of a homogeneous racial and socioeconomic group is a strength, although it limits generalizability of the study. Solid food was introduced at the same time and food was provided by investigators, and there were similar proportions of subjects in the formula groups receiving solid food. Weaknesses include the lack of a concurrent breast-fed comparison group and the small sample size. There could have been selection bias in women who elected to formula feed, and there was no adjustment for potential confounders.

Utility (Adequacy) for CERHR Evaluation Process: This study is of limited utility, except for the bone mineral density portion of the study, which may be of high utility, despite very small sample size.

Stettler et al., 2005 ***(531)*****,** supported by the NIH and the International Formula Council, evaluated the relationship between rate of weight gain in early life and overweight in adults. Subjects included adults (20–32 years old, median age 26 years) who had participated in infant feeding studies between 1965 and 1978 in Iowa. The feeding studies had been restricted to infants who were not breast-fed at all. In these studies, assignment was made to whichever cow-milk or soy formula was being studied in the research unit at the time of the child's birth. Infants were enrolled at 8 days of age and were followed to 112 days of age. The adults who were subsequently recruited for follow-up had consumed their assigned formula for the duration of the study period. Additional foods were permitted after 28 days of age. From the 952 children in the original infant studies, 653 adults were located, met inclusion criteria, and agreed to participate. The subjects were contacted by telephone. Information was obtained from the subjects on current height and weight and on whether the parents of the subjects were overweight. The adult subjects were considered overweight if their body-mass index was ≥25 kg/m^2 and obese if their body-mass index was ≥30 kg/m^2. **[Because there were not enough obese subjects for planned analyses, the analyses involved overweight subjects, who represented 32% of the cohort.]** Infant weight had been obtained at 7 intervals through 112 days of age and was expressed corrected for age as a *z*-score. The period between birth and 8 days of age was identified as the most sensitive for predicting adult overweight, and subsequent analyses focused on this time period. The relationship between adult overweight and weight gain during this period was evaluated by multiple logistic regression with respect to potential confounders.

The unadjusted analysis showed absolute weight gain during the first week of life and from birth to 112 days to be significantly associated with adult overweight. Use of soy formula was not significantly associated with adult overweight in this analysis. With adjustment for birth weight, overweight parents, age in adulthood at the time of the survey, subject income, and first-week infant weight gain, there was a significant association between use of soy formula and adult overweight (OR 1.47, 95% CI 1.01–2.13, *P*=0.046). When infant weight gain from birth to 112 days of age was included in the model, statistical significance was lost (OR 1.37, 95% CI 0.95–1.98, *P*=0.1). The authors called the association between soy formula and overweight an incidental finding and suggested that it be treated with caution due to the inconsistency in results between the analytic models and due to the borderline level of statistical significance. They concluded that "soy-based formulas should be further investigated as a possible risk factor for overweight."

Strengths/Weaknesses: Strengths are that infants were exclusively formula-fed for at least 28 days and were quasi-assigned, i.e., "not strictly randomized," to soy or cow milk by site rather than by mother or pediatrician preference. The multivariate analyses addressed a variety of other factors in infancy that could have been important predictors of adult overweight. If soy formula really is associated with adult overweight relative to cow-milk formula, adjusting for the higher infant weight gain up to 112 days of age (the intervening variable) in the multivariate model would have removed some of the effect of soy formula. Weaknesses are the lack of information on other potential confounders that could have been important in the 26 years to follow-up but weren't measured, the lack of specific information on solid food that was given between 28 and 112 days, and the lack of information on the reasons for mothers selecting exclusive formula feeding (although this should have no bearing on obesity as an outcome). Infants usually lose weight in the first week and regain their birth weight by 2 weeks of age, so weight gain during the first week is not a usual observation in clinical practice.

Utility (Adequacy) for CERHR Evaluation Process: This study is of no utility for the evaluation process.

Venkataraman et al., 1992 ***(532)***, supported by Ross Laboratories, evaluated growth and bone mineral content in 56 healthy term infants exclusively fed human milk or 1 of 2 study formulas until 4 months of age, when baby foods were added to their diets. Infants receiving human milk were also given vitamin D 400 IU daily. Seventeen infants received human milk, while the remaining infants were randomly assigned to cow-milk formula (Similac®, n=19) or soy formula (Isomil®, n=20) using a block for equal sex distribution. The soy formula included modifications from previous soy formulas in calcium source and suspension characteristics. The feeding groups did not differ at the beginning of the study in weight, length, or head circumference. Infants were evaluated at 8, 16, and 24–26 weeks of age for length, weight, head circumference, bone mineral content (single photon absorptiometry of the distal radius), and serum concentrations of calcium, magnesium, phosphorus, alkaline phosphatase, and parathyroid hormone. Data were analyzed using chi-squared for discrete variables and ANOVA or non-parametric methods for continuous variables. The Tukey Student range test was used to evaluate differences between pairs.

No significant differences were detected between the 3 groups in growth measurements or in serum concentrations of calcium, magnesium, alkaline phosphatase, or parathyroid hormone at any time point. Bone mineral content and bone width were significantly greater in the soy group than in the human-milk group at 16 weeks, and the difference in bone mineral content remained significant at 24–26 weeks. Although serum phosphorus levels were lower in the human-milk group and in the cow-milk formula group at 8 weeks, differences did not remain significant at the 16- or 24–26-week evaluation times.

The authors suggested that decreased bone mineral content seen in previous studies in infants receiving soy formula could be attributed to decreased delivery of calcium salts to the infants due to sedimentation. They also suggested that the increased bone width and bone mineral content in soy formula-fed infants compared to breast-fed infants could be a result of lower nutrient density of human milk. The researchers concluded that the decreased bone mineralization associated with soy-formula feeding in infants could be prevented through improved suspension characteristics of the minerals used and also noted the importance of these characteristics in the interpretation of studies involving bone mineral status.

Strengths/Weaknesses: Strengths include the use of 3 feeding groups including a breast-fed group, the random assignment of formula-fed infants, with procedures for assuring approximate equal distribution by infant sex, the multiple measurements of growth including head circumference measured and bone mineral content, and the restriction of solid foods during most of the study period. There were, however, apparently no adjustments for confounders. Selection bias may have occurred for breast-fed infants, and the sample was small with 17–20 in each group. There was a difference in milk consumption between the groups.

Utility (Adequacy) for CERHR Evaluation Process: This study is of limited utility in the evaluation process.

3.2.1.2 Gastrointestinal effects

Reports on gastrointestinal effects of soy formula are presented. Case series are followed by controlled studies. Some reports include what may be gastrointestinal manifestations of allergic disease, and these studies might just as reasonably have been discussed in **Section 3.2.1.3**.

Ament and Rubin, 1972 ***(533)***, supported by the NIH, the Public Health Service, the Children's Orthopedic Hospital and Medical Center, and the University of Washington, presented a case of a 6-week-old infant with an immediate response to soy feeding consisting of fever, leukocytosis, cyanosis, vomiting, blood-tinged mucoid diarrhea, dehydration, and metabolic acidosis, all of which disappeared after 3 days of iv feeding. Subsequent challenges with soy protein were associated with violent systemic symptoms and jejunal abnormalities on endoscopy consisting of loss of villi and surface ulcerations. There was no reaction to gluten, soy lecithin, lactose, or sucrose. A moderate reaction to cow milk disappeared by 10 months of age, whereas the soy reaction continued to occur. The authors suggested that this response was unlikely to be allergic. They noted a similarity of the intestinal lesion to that of celiac disease and wondered if the underlying mechanism of injury might similarly be based on a genetic abnormality.

Strengths/Weaknesses: This paper represents a single case report of what may be a unique reaction.

Utility (Adequacy) for CERHR Evaluation Process: This report is of no utility in the evaluation process.

Burks et al., 1994 ***(534)***, supported by NIH and Ross Laboratories, studied 43 infants under the age of 18 months to evaluate whether formula protein source was associated with enterocolitis. All subjects were suspected to have milk- and/or soy-protein enterocolitis due to vomiting or other gastrointestinal symptoms. Patients with suspected IgE-mediated adverse food reactions were excluded. Formula challenges were performed in the hospital when the children were symptomatic. The challenge groups were:

Group 1 (n=23): cow-milk formula, powdered soy formula, liquid soy formula,
Group 2 (n=12): cow-milk formula, liquid soy formula, powdered soy formula, and
Group 3 (n=8): cow-milk formula, then soy formula challenges in randomized order.

[The grouping was not part of the original study design; study investigators modified the order of formula administration after results from the first group failed to support their hypothesis that the last formula would be most likely to produce a reaction. Thus, testing of the 3 groups was successive, and only the last group had a randomization component.]

The subjects fasted for 4–6 hours prior to challenge, and there were 48 hours between challenges. Patients were observed and stools were collected during the 24 hours following challenge. Reactions were considered positive if 2 or more of the following criteria were met: 1.) vomiting or diarrhea developed; 2.) absolute neutrophil count increased 6–8 hours after the challenge by at least 3500/mm^3 compared to blood drawn immediately before challenge; 3.) blood was identified in stool; 4.) leukocytes were present in Wright-stained fecal mucus; and 5.) Charcot-Leyden crystals or eosinophilic debris were noted in stool after Hansel staining. Laboratory personnel were blinded to formula type. Statistical analysis methods were not given in detail. **[Subjects appear to have been combined without regard to group.]**

Twenty-one infants (48.8%) had a negative challenge to milk and soy formulas, 12 (27.9%) reacted only to soy, 4 (9.3%) reacted only to cow milk, and 6 (14.0%) reacted to both cow milk and soy. **[Statistical comparisons were not reported; chi squared test by CERHR gives *P*=0.1 for any soy reaction compared to any cow-milk reaction.]** Patients who reacted to only 1 type of soy formula tended to react to whichever formula was administered first. Because the order of administration rather than the type of

protein isolate was associated with enterocolitis, the authors concluded that processing soy into a powdered form did not render it less likely to cause a reaction. The authors interpreted the ordering effect as evidence that milk- and soy-protein enterocolitis may be caused by an unknown local immune mechanism with depletion of gastrointestinal immune mediators by the first challenge, regardless of protein source.

Strengths/Weaknesses: Strengths include the evaluation of all infants with enterocolitis thought to be due to formula intolerance and sequential administration of three types of protein. The non-random order of the challenge sequence and the lack of accounting for formula type at baseline are weaknesses.

Utility (Adequacy) for CERHR Evaluation Process: This report has no utility in the CERHR evaluation process.

Halpin et al., 1977 ***(535)***, funding not indicated, reported 4 infants, aged 2–4 months, with suspected intolerance to soy protein. Infants were admitted to the hospital, and proctosigmoidoscopy with rectal biopsy was performed within 24 hours. Gross and microscopic evidence of acute colitis was present in all 4 infants. After 1 month, all infants had recovered and sucrose and lactose tolerance tests were normal. Proctosigmoidoscopy with biopsy was repeated to confirm mucosal normalcy. Soy-protein challenge was given by feeding increasing amounts of soy formula (Isomil®) over 8 hours. If the feeds were tolerated, the soy formula was continued every 4 hours for up to 5 days. Stools were weighed and tested for occult blood and for the presence of reducing sugars. If the stool results were abnormal, a proctosigmoidoscopy with rectal biopsy was performed. Soy-protein challenge produced symptoms in the infants within 3 hours to 5 days. Proctosigmoidoscopy revealed friable mucosa, loss of vascular pattern, acute colitis (polymorphonuclear leukocytes present in the lamina propria or in the walls of the rectal glands), and an increase in mitotic cells in the crypts. Other findings included frank mucosal hemorrhage (2 infants), crypt abscess (1 infant), and depleted rectal-gland mucus (2 infants). Abnormal stool tests (low pH and increased reducing sugars) occurred in 2 infants, but these infants had no symptoms and their biopsies were normal. The authors suggested that the normal biopsies could be a result of rapid recovery, mild infection, or patchy mucosal damage. When biopsies from symptomatic children were evaluated blindly against biopsies from children with other colitides, the authors were unable to distinguish infectious colitis or mild ulcerative colitis from soy protein-induced colitis. They were able to distinguish severe ulcerative colitis and granulomatous colitis, nodular lymphoid hyperplasia, and normal bowel from the specimens taken in this study. The authors concluded that soy protein-induced intestinal mucosal damage is under-reported, and that soy protein may have the same potential for mucosal injury as does cow-milk protein.

Strengths/Weaknesses: In this case series, all 4 infants were known to have been fed soy formula and were suspected to be intolerant. The withdrawal and soy protein challenge under controlled conditions is a strength, but there was no comparison group. This population may represent a uniquely susceptible subgroup.

Utility (Adequacy) for CERHR Evaluation Process: This report is of no utility in the evaluation process.

Iyngkaran et al., 1988 ***(536)***, funding not indicated, investigated the effects of soy protein on the small bowel mucosa of 18 infants who suffered from acute gastroenteritis. Infants were hospitalized

for correction of fluid and electrolyte status, following which they received a protein hydrolysate formula (such as Pregestimil®, Nutramigen®, Alfare®, or pre-hydrolyzed expressed breast milk) for 6–8 weeks. Infants were readmitted again for soy-protein challenge (Prosobee®) and evaluated by biopsy for histologic appearance and enzyme levels in the jejunal mucosa before and 20–24 hours after the challenge. Ten (55.5%) of the 18 infants developed soy-protein enteropathy. The infants were divided into 3 groups based on their histologic and clinical results. Group 1 (n=3) demonstrated histologic and clinical reactions between the 3rd and 9th hour of soy-protein challenge. Histologic reactions included depletion of the mucosal enzymes lactase, sucrase, maltase, and alkaline phosphatase and depressed blood xylose levels; clinical reactions were diarrhea, weight loss, vomiting, lethargy, fever, rhinitis, and skin rash. In Group 2 (n=7), infants experienced only histologic reactions to the initial challenge. As a group, they had less depression of mucosal enzyme activity and blood xylose absorption compared to Group 1, but individual reactions varied considerably. Group 3 (8 infants) did not have any histologic, enzymatic, or clinical reactions to the soy-protein challenge. Significant differences in mucosal enzymes and xylose absorption were not found on comparison of Groups 1 and 2 to Group 3.

Strengths/Weaknesses: The clinical evaluation after soy challenge and the inclusion of biopsies are strengths, but this sample is highly select group of children with acute gastrointestinal illness apparently due to a variety of causes. It was not possible to determine exactly which of the 18 infants had been previously exposed to soy prior to the acute episode and soy challenge. There was no comparison group.

Utility (Adequacy) for CERHR Evaluation Process: This report is of no utility in the evaluation process.

Lothe et al., 1982 ***(537)***, funding not indicated (but formula provided by Mead Johnson), carried out a double-blind crossover study to determine the effect of cow milk on infantile colic. Sixty-five cow-milk formula-fed infants with symptoms of infantile colic were identified at the Children's Hospital in Malmö, Sweden and enrolled. Of these, 60 infants between the ages of 2 weeks and 3 months completed the study. Identical-appearing cans of formula were provided to the parents for use over a 2-week period. The cans contained 1 week of cow-milk formula (Enfamil®) or 1 week of soy formula (ProSobee®). **[Randomization of order was not discussed.]** Five infants also received some human milk, and their mothers were instructed not to drink cow milk during the study period. Parents completed a daily questionnaire noting symptoms of colic (including paroxysmal abdominal pain, sustained severe crying, distended abdomen due to gas, and frequent wish to suck). Parents were also asked to note "whether the infant seemed to thrive" on the test formula. Infants who appeared to have improved with soy the first week and then worsened the second week on cow milk were put back on soy formula at the end of the study period. Infants who received soy formula the second week and appeared to improve were given a cow-milk formula challenge 1, 3, and 9 months later to exclude spontaneous recovery. Infants whose symptoms did not improve with either the soy or cow-milk formulas were then given a hydrolyzed casein formula (Nutramigen®), a casein hydrolysate based on cow's milk protein. Infants who improved on the casein hydrolysate formula were also rechallenged with cow-milk formula **[results not discussed here]**.

Seventeen infants (29%) had what appeared to be a spontaneous recovery and tolerated the cow-milk formula. In 11 infants (18%) who did not tolerate cow-milk formula, colic disappeared within 48 hours

of being put on soy formula. On rechallenge with cow milk 1 month later, 8 of the 11 infants had recurrent symptoms. Of the remaining 32 infants (53%) whose symptoms did not diminish on either cow-milk or soy formula, all were symptom-free within 48 hours of being placed on casein hydrolysate formula, and 14 of the 32 infants had recurrent symptoms when rechallenged with cow milk.

The authors concluded that cow milk is a significant factor in the development of infantile colic in formula-fed infants. They hypothesized an allergic cause and suggested dietary treatment with alternative formulas, such as those based on soy or casein hydrolysate.

Strengths/Weaknesses: All infants were said to have severe colic and to have been fed with cow milk-based formula. Infants were treated with both cow-milk and soy formula successively, with the mother blinded to formula content. To rule out spontaneous recovery, infants who recovered were challenged again later with cow milk formula. It was unclear, however, how formula was assigned and if it made a difference which formula was given first. Later challenges to rule out spontaneous recovery were only performed with cow milk even if the infant had not improved on soy formula. In addition, there was no inclusion of a group of infants without colic.

Utility (Adequacy) for CERHR Evaluation Process: This report is of no utility in the evaluation process.

Poley et al., 1983 ***(538)***, supported by NIH, reported observations from scanning electron microscopy and light microscopy of small intestine biopsies from 2 infants with soy protein-induced villous atrophy. The first biopsy was performed on admission, and a follow-up biopsy was performed 6 weeks later. Mucosa obtained from a 7-month-old infant who had been on a sucrose-restricted diet with no diarrhea for 4 weeks preceding biopsy was used as a control. The soy-sensitive patients showed flattening of the villi, indistinct cell borders, lack of glycocalyx, and exposed microvilli. The degree of villous atrophy was similar to that seen in gluten-sensitive enteropathy. The authors estimated that 50–75% reconstruction of villous height had occurred at the 6-week follow-up biopsies. Tissue disaccharidase activity **[measured as the production of glucose after incubation of disaccharide with a tissue homogenate]** was depressed during soy feeding but showed recovery after 6 weeks. The authors described the recovery after cessation of soy exposure as "remarkable."

Strengths/Weaknesses: The detailed evaluation of the clinical and histological picture of the disease and course of recovery is a strength, as is the inclusion of a control patient. It is a weakness that only 2 infants were included and that information related to age, feeding regimen during the entire period and other medical facts were not known.

Utility (Adequacy) for CERHR Evaluation Process: This report has no utility for the CERHR evaluation process.

Powell, 1978 ***(539)***, support not indicated, reported 9 infants, aged 4–27 days at onset of symptoms, with protracted diarrhea before and after milk and soy-formula challenges. A control group consisted of 11 infants (age 2–9 months) who were hospitalized for chronic diarrhea not caused by cow-milk or soy formula. **[Not all of these control infants were tested in the same manner as the formula-intolerant infants, and results for the control infants are not clearly presented.]** Infants in both

groups were managed with iv therapy if needed and with a casein hydrolysate formula (Nutramigen®). Once normal stools were observed and appropriate weight was gained, the children were challenged with cow-milk formula and, at least 1 week later, with soy formula. Stools were tested 12 hours prior to the challenge and 48 hours after the challenge. Complete blood counts, serum β1C globulin, and serum protein electrophoresis were determined before and after the challenge. A positive response to the challenge was defined as liquid stools or stools that contained blood or leukocytes within 24 hours of the challenge. In the group with formula intolerance, 8 of 9 infants had positive responses to the cow-milk challenge and 6 of 9 had positive responses to the soy challenge. Serum protein electrophoresis remained unchanged in all but 1 infant. There was a statistically significant increase in serum β1C globulin levels after the positive responses 90 minutes after the challenge but not thereafter. Leukocytosis occurred 6–10 hours after the positive challenges. No changes in eosinophils (<6% of white blood cell count), hemoglobin, or hematocrit values were observed.

The authors concluded that the severity of responses may have been unique to this age group. They also concluded that soy formula can be just as damaging as cow-milk formula if fed during this stage in the infant's life or after a reaction to cow milk.

Strengths/Weaknesses: This paper included infants with known cow-milk or soy protein gastrointestinal sensitivity. A comparison group with similar symptoms that were known not to be due to formula was used, and challenge with both cow-milk and soy formula occurred in each child. Weaknesses include the apparent non-random order of challenge (cow milk and then soy) and it was not clear if the control group received the same challenges and what the results were.

Utility (Adequacy) for CERHR Evaluation Process: This report is of no utility in the evaluation process.

Ostrom et al., 2006 ***(540)***, supported by Abbott laboratories, evaluated the effect of a high-fiber soy formula on regurgitation in healthy infants whose parents reported that more than 25% of feedings were associated with regurgitation. Formula-fed infants were enrolled at 13–32 days of age and randomized to receive a cow-milk formula (Similac® With Iron, n=90) or a soy formula with 6 g added soy fiber (Isomil® DF, n=89. Parents recorded the incidence and estimated volume of regurgitation during the first 7 days and the last 3 days of a 28-day feeding period. Study completion rates were 74–76% with formula intolerance as the most common reason for discontinuation. The number of regurgitation episodes decreased in both groups; however, the soy formula-fed group had fewer daily regurgitations at 7 days and at 28 days than the cow-milk formula group. The mean ± SEM number of daily regurgitations went from 3.6 ± 0.2 at baseline to 2.4 ± 0.3 on study day 28 in the cow milk-formula group and from 3.9 ± 0.2 at baseline to 2.0 ± 0.2 in the soy-formula group.

Strengths/Weaknesses: The study design and sample size were adequate; however, the inclusion and exclusion criteria based on history were vague. Infants were excluded for a history of pyloric stenosis, but pyloric stenosis may not become evident until 4–6 weeks of age. The volume of regurgitation was not well quantified, but may have been inconsequential if all infants thrived. Length and head circumference measurements would have been helpful in this regard. It was unclear if the 10% of women who refused baseline measurement and the 25% of remaining women who did not complete this relatively short trial influenced the results. It is curious that in study Figure 2, there appears

to be a significant difference in regurgitation frequency between treatment groups on day 1 of the intervention.

Utility (Adequacy) for CERHR Evaluation Process: This paper is of no utility in the evaluation process.

3.2.1.3 Allergy and Immunology

Agostoni et al., 2007 *(541)*, support not indicated, conducted a prospective study to compare growth in infants with immunoglobulin E-mediated cow's milk allergy who were fed breastmilk or randomly assigned to drink one of three special formulas during the complementary feeding period of 6 to 12 months. A total of 160 male and female infants entered the study in the period from June 2001 to December 2005 at the San Paolo Hospital in Milan, Italy. Of the 160 enrolled, 125 infants completed the study requirements. The three formulas included in the study were soy formula (n=32), a casein hydrolysate (n=31), and a rice hydrolysate (n=30). Breastfed infants (n=32) were considered the reference group. Eligibility criteria include being fully breastfed for the first 4 months of life and a diagnosis of cow's milk allergy by 6-months of age. Groups were compared for weight-for-age (WA), length-for-age (LA), and weight-for-length (WL) z-scores at 6, 9, and 12 months of age. The primary outcome measure was the differences in WA, LA, and WL *z*-scores between 6 and 12 months. Power analysis indicated that 29 subjects per group were required to detect a +0.4 relative change difference in weight-for-age z-score. Statistical analysis of continuous variables was conducted with ANOVA followed by *post-hoc* bivariate analysis (Student's *t* test) and Bonferroni's adjustment. Multivariate analysis was performed with ANOVA. Discrete variables were analyzed with chi-square test or Fisher's exact test.

Authors reported that infants with cow's milk allergy had lower growth parameters at 6 months **[comparison group not stated but assumed to be infants without cow's milk allergy or atopic dermatitis]**. The growth parameters of WA, LA, and WL *z*-scores change between 6-12 months did not differ between the breastfed infants and any of the formula groups. The overall analysis yielded a significant difference in the 6-12 month WA change among the groups. Based on uncorrected bivariate comparisons, the 6-12 month change in WA *z*-score for the soy formula group (-0.16) was significantly lower compared to the rice (0.18, P=0.019) and casein (0.16, P=0.029) groups However, after Bonferroni's adjustment, the significance levels for these comparisons were 0.090 and 0.120, respectively. Multivariate analysis indicated that feeding group and acceptance of the dietary schedule ("good", "average", or "poor" meal consumption) at 12 months were significantly related to changes in the WA *z*-score. Based on these findings, the authors concluded that infants fed hydrolyzed products showed a trend toward higher WA z-score increments in the 6- to 12-month period indicating better short-term weight gain compared with soy formula. However, the clinical significance of these differences in weight gain are unknown given that no growth parameter in the formula group differed from breastfed infants.

Strengths/Weaknesses: Strengths included breastfeeding for 4 months and then assignment to the formulas. Weakness: no mention of maternal restrictions during breastfeeding, diagnosis of cow milk protein allergy.

Utility (Adequacy) for CERHR Evaluation Process: This study is of limited utility for the evaluation.

Chandra et al., 1989 ***(542)***, supported by the National Health Research Development Programme of Health and Welfare Canada, and the Mead Johnson Canada division of Bristol Myers, examined the effects of maternal diet during breast feeding and the effects of 3 different types of formula on the development of atopic eczema in infants with family histories of atopic disease. **[Dr. Ranjit Chandra has been accused of scientific fraud related to a number of publications. Because of these charges, the AAP Committee of Nutrition has deleted references to Dr. Chandra's work from as many of its current publications as possible.]** A mother was recruited if either she or the baby's father had a family history of atopy. Mothers planning to breast feed exclusively were randomly assigned to an unrestricted-diet group or an experimental group asked to exclude dairy products, eggs, fish, peanuts, and soybeans from their diet and to take a 1 g calcium supplement daily for 6 months or the duration of lactation. Compliance with the requested restrictions was assessed by questioning, analysis of food diaries, and testing for β-lactoglobulin and ovalbumin in random samples of human milk. Mothers who did not plan to breast feed were randomly given either cow-milk formula (Enfalac®, n=40), soy formula (Prosobee®, n =41), or casein-hydrolysate formula (Nutramigen®, n=43), which they were told to feed the infant for at least 6 months. The mothers and examiners were unaware of the formula type. Infants were examined by a physician at 2, 4, 6, 12, and 18 months, and an eczema score was constructed based on affected parts of the body, type of eruption, and severity. Student *t* test was used to compare square-root transformed eczema scores, and proportions were compared using the chi-squared test with Yates correction. The 3 formula groups were compared using the Tukey *W* test for transformed scores and chi-squared test for proportions.

Among breast-fed infants, maternal dietary restriction was associated with a lower mean eczema score and a lower proportion of affected infants **[not further discussed for the purposes of this report]**. A comparison of the 3 formula-fed groups shows a lower proportion of affected infants and lower eczema scores in the group given the casein hydrolysate than in the other 2 groups. Only 9 infants in the casein-hydrolysate group developed eczema, while 36 infants in the soy group and 38 infants in the cow-milk group developed eczema. No difference was detected in the incidence of eczema between the soy formula and cow-milk formula groups. The authors recommended that formula-fed infants with family history of atopy be given a casein hydrolysate formula.

Strengths/Weaknesses: Strengths include use of a homogenous group with positive family history of atopic disease, random assignment to 1 of 3 formula groups, 1 of which was restricted to soy formula for at least 6 months, randomization after maternal selection of breast or bottle, blinding of mothers and observers to formula type, and separate analysis of the formula group due to self-selection. Weaknesses include the relatively small sample size of soy formula-fed infants (n=41) and the lack of mention of introduction of other foods. There were no multivariate analyses, and although baseline characteristics were said not to differ, no statistical comparisons were made.

Utility (Adequacy) for CERHR Evaluation Process: This study is of no utility for the evaluation because the scientific integrity cannot be confirmed.

Chandra et al., 1989 [543; 544], supported by the National Health Research Development Program of Health and Welfare Canada, Carnation Nutritional Products, and the Nutrition Research Education Foundation, conducted a prospective randomized controlled study to examine the effects of exclusive breast feeding and the feeding of 1 of 3 different types of formula on the incidence of atopic disease in predisposed infants. Mothers were recruited if either they or the baby's father had a family history

of atopy. Mothers planning to breast feed (n=60) were instructed to breast feed exclusively but were not given any other instructions regarding their own diets. Mothers who did not plan to breast feed were randomly given either cow-milk formula (Similac®, n=24), soy formula (Isomil®, n=25), or whey-hydrolysate formula (Good Start H.A.®, n=68), which they were told to feed their infants exclusively for 6 months. The mothers and examiners were unaware of formula assignment. After 6 months, families were given usual advice for the feeding of infants with family history of allergy. Infants were examined at 6, 12, and 18 months of age for clinical manifestations of possible allergy. Skin tests were conducted on symptomatic infants using commercial antigens, and IgE antibodies to milk and soy were measured using commercial kits.

Infants in the whey-hydrolysate group had significantly lower incidence of atopic symptoms than those in the cow-milk and soy-formula groups. No significant differences were apparent between the cow-milk and soy-formula groups or between the whey-hydrolysate and breast-fed groups. Serum IgE antibodies to cow-milk or soy proteins were described as more common in infants fed the respective protein source **[statistical comparisons not reported]**. Skin prick tests in symptomatic infants did not identify significant differences between cow milk- and soy formula-fed infants. The authors concluded that there was a lower incidence of atopy in predisposed infants who were breast-fed or fed hydrolysate compared to predisposed infants fed cow-milk or soy formula.

Strengths/Weaknesses: Strengths include use of a homogeneous group with positive family history and randomization to formula groups after the maternal decision not to breast feed. The use of 3 formula groups with exclusive feeding for 6 months and the blinding of mothers and observers to formula type are additional strengths. The sample sizes were moderate with only 25 subjects in the soy-formula group. Breast feeding mothers were self-selected. There was no mention of solid foods, and although baseline characteristics were said to not differ, there were no multivariate analyses.

Utility (Adequacy) for CERHR Evaluation Process: This study is of no utility for the evaluation because the scientific integrity cannot be confirmed.

Chandra et al., 1997, 1998 [545; 546] presented 5-year results from the above study. The prevalence of eczema by 60 months was 6/60 **[10%]** in the breast-fed group, 10/68 **[15%]** in the whey-hydrolysate group, 19/68 **[28%]** in the soy-formula group, and 20/67 **[30%]** in the cow-milk formula group. The prevalence of asthma by 60 months was 4/60 **[7%]** in the breast-fed group, 7/68 **[10%]** in the whey-hydrolysate group, 14/68 **[21%]** in the soy formula group, and 16/67 **[24%]** in the cow-milk formula group. Skin prick testing and double-blind placebo-controlled food challenges showed a lower incidence of food allergy in the whey-hydrolysate and breast-fed groups. The authors concluded that breast feeding is preferred in children with a high risk of atropy, and that among formulas, the whey-hydrolysate formula was preferred to cow-milk or soy formula. **[The Expert Panel notes that the whey-hydrolysate formula was marketed by Carnation, a sponsor of this study.]**

Strengths/Weaknesses: This 5-year follow-up has the same strengths and weaknesses as the earlier reports *(543; 544)*.

Utility (Adequacy) for CERHR Evaluation Process: This study is of no utility for the evaluation because the scientific integrity cannot be confirmed.

Cordle et al., 2002 ***(547)***, supported by the Ross Products Division, Abbott Laboratories, reported additional results from the trial reported by Ostrom *et al.*, 2002 *(548)*. No feeding-group differences were detected in total white blood cell count or in numbers or percentages of the 5 major leukocyte populations; however, at 6 months, the breast-fed group had significantly higher numbers and percentages of eosinophils than the group given nucleotide-supplemented soy formula. The breast-fed group also had a higher percentage of lymphocytes at 12 months than the group given nucleotide-supplemented soy formula. No feeding-group differences were detected in numbers or percentages of B, pre-B, or mature B lymphocytes. No significant differences were identified in the numbers or percentages of total T, helper T, or cytotoxic/suppressor T cells between the breast-fed group and the group given unsupplemented-soy formula. At 7 months, the group given supplemented-soy formula had a higher percentage of total T lymphocytes, higher numbers and percentages of helper T lymphocytes, and higher percentages of non-natural killer-like T cells. Despite the differences, all values were reportedly within normal ranges.

The researchers were unable to detect feeding-group differences in expression of the human leukocyte antigen or in the maturation state of helper T lymphocytes, with the exception that the supplemented-soy group had higher percentages of naïve helper T cells than the breast-fed group at 6 and 7 months and a higher number of memory/effector helper T cells than the breast-fed group at 7 months. The study authors reported that the concomitant increase in CD45RA and CD45RO cells suggested a balanced, antigen-driven conversion of the helper T-cell population. No differences were detected in numbers or percentages of natural killer lymphocytes or natural killer T lymphocytes between the 3 feeding groups, with the exception of the supplemented soy group having lower numbers and percentages of natural killer cells compared with the breast-fed group and the unsupplemented soy group having lower percentages of natural killer T cells than the breast-fed group at 12 months.

The authors concluded that there were no consistent significant differences in immune status, maturation, or level of immunocompetence between infants fed a soy-based formula and those fed human or cow milk. They also reported that their study failed to demonstrate a strong effect of nucleotide supplementation of soy formula on immune status of infants.

Strengths/Weaknesses: Strengths and weaknesses are similar to those for Ostrom *et al.*, 2002 *(548)*.

Utility (Adequacy) for CERHR Evaluation Process: This study is of limited utility for the evaluation process.

Fort et al., 1990 ***(549)***, funding not indicated, conducted a retrospective study of 59 children with autoimmune thyroid disease, their 76 healthy siblings, and 54 healthy, unrelated control children to determine whether feeding soy formula or breast feeding was associated with the development of thyroid disorders. The children with autoimmune thyroid disease included 52 with Hashimoto's thyroiditis and 7 with Graves' disease. A nutritionist evaluated the history of infant feeding early in life, including the type of formula given and the age at which solid food was introduced. Proportions were evaluated using a chi-squared test. No significant differences were seen in the prevalence or duration of breast feeding or in the age at which solid foods were introduced. Among children with thyroid disease, there were no significant differences in the initial serum values of thyroxine or thyroid-stimulating hormone or in anti-thyroid antibody titers between breast-fed and bottle-fed

infants **[data not shown]**. Of children with thyroid disease, 31% had received soy formula as infants compared to 12% of healthy siblings ($P<0.01$) and 13% of healthy unrelated controls ($P<0.02$). The authors concluded that they were unable to document an advantage of breast feeding with regard to the subsequent development of autoimmune thyroid disease, The greater prevalence of soy-feeding among children with thyroid disease compared to children without thyroid disease was proposed as being due to a possible decrease in cow-milk tolerance among children predisposed to developing thyroid disease or to possible adverse effects of soy on the developing thyroid.

Strengths/Weaknesses: The use of sibling controls plus a healthy unrelated control group is a strength. Weaknesses include the retrospective data collection and the small sample. As the authors suggested, the reasons for selecting soy formula may have confounded the association between this product and subsequent thyroid disease. This study cannot make that distinction.

Utility (Adequacy) for CERHR Evaluation Process: This study is of no utility for the evaluation process.

Gruskay *(550)*, support not indicated, conducted a prospective evaluation of 908 children, 328 of whom had a family history of major allergy, and a control group of 580 children with no such family history, with the objective of determining if diet during the early months of life was related to the development of atopy later in childhood. The study population consisted of consecutive, healthy full-term newborns under the care of the author or participating pediatricians. In the families with a history of allergy, breast feeding was chosen by the mothers of 48 subjects. The remaining infants were assigned non-randomly to soy formula (every fourth child, n=79) or cow-milk formula (n=201) **[formula composition not given].** Children were evaluated every month from 1 to 6 months of age, then every 2 or 3 months until age 2, and every year for up to 17 years thereafter. Children without a family history of allergy all received cow-milk formula if bottle-feeding was elected by the mother. Comparisons were made in the proportion of children at different ages with allergy. Statistical procedures were not discussed.

After 15 years, 50% of infants with a family history of allergy had developed allergic disease, while only 15% of control infants had developed allergy **[level of significance not given**]. In infants with a positive family history, allergy developed over 15 years with the same frequency in those fed cow-milk formula as in those fed soy formula (53%). Allergy was present significantly less often in children with a family history of allergy if they had been breast-fed, but differences in allergy rates between bottle- and breast-fed infants did not become statistically significant until after 3 years of age. The author concluded that breast feeding may play a role in decreasing the incidence of allergic disease. He also concluded that soy formula has no apparent advantage over cow-milk formula in the prevention of allergy.

Strengths/Weaknesses: Strengths include the large sample size with both positive and negative family history groups. There was some attempt to randomly assign mothers in the positive family history group to soy or cow-milk formula groups. The long-term follow-up is a strength, although the statistical methods did not include time in follow-up. The authors appeared to use appropriate denominators at each follow-up time point. Weaknesses include the possible selection bias inasmuch as mothers could elect to breast feed. Mixed feeding was allowed, and there was no adjustment for

other foods when introduced. In the end, it appears authors only compared soy formula-fed to breast-fed infants in the positive family history group. Soy formula was not formally compared to cow-milk formula, although rates of allergy incidence were similar overall in both soy and cow-milk formula groups. There was no adjustment for potential confounders.

Utility (Adequacy) for CERHR Evaluation Process: This study's utility is limited to a comparison of soy formula feeding and breast feeding in infants with a positive family history.

Halpern et al., 1973 ***(551)***, supported by Borden, Inc., followed 1753 Caucasian infants (893 male) who were fed either human milk, soy formula, or cow-milk formula from birth to 6 months of age to observe the influence of diet, family history, and sex on the development of childhood allergy. Assignment to the 3 dietary groups was not randomized, but was as "unprejudiced as possible if the mother did not insist on a particular regimen." Breast feeding occurred in 352 infants, soy formula was given to 317 infants, and cow-milk formula was given to 1081 infants. The soy formula was Mull-Soy®, and the cow-milk formula was a proprietary liquid formula (Bremil®, Similac®, Enfamil®, or SMA®). The mothers of the breast-fed infants were advised to limit their own cow-milk intake to 1 pint per day. The infants were followed for varying periods of time up to age 7 years. Diagnosis of food allergy was made solely on clinical grounds, with at least 3 episodes of characteristic symptoms being required to verify each diagnosis. Of the 1753 children, 401 were diagnosed as either definitely or possibly allergic. Children with a family history of allergy were significantly more likely to develop allergy, but diet had no significant effect on the development of childhood allergy. The authors noted that only 0.5% of infants fed soy milk developed allergy in the first 6 months of life, while 1.8 % of infants fed cow milk became allergic to it ($P<0.05$, chi-squared). The authors concluded that this finding agreed with the conclusions of other investigators that "soy milk is a less potent sensitizer."

Strengths/Weaknesses: Strengths include a large sample size from 9 different pediatric offices with 3 feeding groups and prospective ascertainment and follow-up for as long as 7 years. The authors evaluated a broad spectrum of allergic endpoints and included family history. There was, however, a lack of randomization to feeding group; women who had a preference could choose method of feeding and more women with immediate family history of allergy chose soy formula. Pediatrician preference also played a role. Many of the groups were mixed-feeding groups and mothers could have changed methods of feeding during the study period. Infants were followed for varying lengths of time, and there was substantial attrition at each follow-up point. No time variable was included in the analyses, and the denominator for the incidence of allergy was the entire sample regardless of the length of follow-up. Not all children were evaluated for allergies by a specialist, only those referred by the pediatrician. Multivariate analysis should have been used to control for multiple potential confounders simultaneously.

Utility (Adequacy) for CERHR Evaluation Process: This report is of no utility in the evaluation process.

Jenkins et al., 1984 ***(552)*** funding not indicated, studied 46 children with colitis diagnosed within a 4-year period. They reported that the age distribution of the children showed peaks at <2 years and 6–13 years, with all 38 children in the older group suffering from either ulcerative colitis or Crohn's disease. The researchers compared the remaining 8 children with selected controls from the

older group. Of these 8 subjects, 6 presented before 4 months and were switched from a cow-milk to a soy-milk diet. The remaining 2 subjects were receiving a mixed diet and were switched to an egg-free cow-milk diet. For each of these 8 patients and selected controls, dietary history, family history of allergy, blood count, serum immunoglobulins, IgE, and IgE antibodies to whole cow milk, β-lactoglobulin, and ovalbumin were studied. Colonoscopies were also performed in 7 of the patients and in the controls. Biopsies were taken before and after the implementation of the exclusion diets and examined for eosinophils and IgE-containing cells.

Symptoms disappeared in all 8 patients when switched to soy formula. Symptoms recurred within 2–6 weeks of the switch in 3 of the 8 patients but resolved after a chicken-based formula was substituted. Two patients who were given cow milk again 9–12 months after diagnosis showed symptoms again within 24 hours. Another patient who was given beef broth at 1 year also showed symptoms. All symptoms resolved after return to the exclusion diet. The researchers found that positive family history was significantly more common in the 8 patients with food-allergy colitis than in the selected controls. Blood eosinophil counts, serum IgE concentrations, and antibodies were also higher in the study group. Colonoscopies of the study group were distinctly different from those of controls, with an increase in eosinophils and IgE-containing cells. The authors concluded that colitis arising during infancy was most likely a result of food allergy and was best treated with an exclusion diet.

Strengths/Weaknesses: It is a strength that biopsies were taken before and after exclusion diets, but a weakness that infants were compared with much older "selected" controls.

Utility (Adequacy) for CERHR Evaluation Process: This study is of no utility for the evaluation process.

Kjellman and Johansson *(553)*, supported by Förenade Liv, Semper Fund for Nutritional Research, Linköping University, and the Swedish Medical Research Council, performed a randomized clinical trial of soy formula (n=23) or cow-milk formula (n=25) in children with biparental atopic disease. Mothers were encouraged to breast feed, and infants were placed on a randomly assigned formula from weaning until age 9 months. At age 9 months, soy formula-fed infants were slowly introduced to cow milk. Children were followed to age 4 years. They were evaluated periodically, and atopic disease was rated as obvious, probable, possible, or none based on the number of episodes of atopic dermatitis, bronchial asthma, allergic rhinitis, allergic urticaria, or gastrointestinal allergy. Cord blood and venous blood drawn at 3, 9, 12, 18, and 36 months of age was analyzed for IgE and IgG antibodies to cow-milk and soybean protein and for levels of IgE, IgG, IgA, IgM, hemoglobin, cholesterol, and albumin. Statistical methods were not discussed.

Obvious and/or probable atopic disease occurred in two-thirds of children during the 4-year follow up period. There was no statistically significant difference in development of obvious or probable atopic disease by sex or formula type. Four children in each formula group were found to have IgE antibodies to cow milk. IgG antibodies to β-lactoglobulin, a protein, were present in the cord blood of all but 3 infants. The cow-milk formula group had significantly higher levels of IgG antibody to β-lactoglobulin from age 3 to 18 months, but the difference was no longer statistically significant at 3 years (**Table 87**. **[The Expert Panel cannot evaluate these data because the graph in the original paper did not indicate variance, and the statistical methods were not given.]**

Table 87. Mean IgG Antibody Levels to β-Lactoglobulin (Kjellman and Johnson, 1979)

Age, months	*Formula*		*P-value*
	Soy	*Cow Milk*	
Newborn	26	38	Not significant
3	28	120	<0.01
9	24	355	<0.001
12	85	270	<0.05
18	74	285	<0.05
36	83	237	Not significant

Data given as mean ‰ of a reference serum, estimated from a graph. The reference serum was not defined, variances were not shown, and the statistical methods were not given.

From Kjellman and Johansson, 1979 *(553)*.

IgE antibodies to soybean protein were not shown to differ significantly between groups. Serum IgG antibodies to soybean protein were significantly higher at 9 and 12 months of age in the soybean formula group: mean ± SD 65.7 ± 164.1 (9 months) and 63.3 ± 104.5‰ (12 months) of reference serum compared to the cow-milk formula group: 9.5 ± 2.0 (9 months) and 24.5 ± 52.5‰ (12 months) of reference serum. No differences in IgE levels were detected between healthy and atopic children, and no significant differences were detected at birth or at 3, 18, or 36 months.

Overall, there were very few side effects of soy-formula intake, and all children in the soy group tolerated the formula, except for 1 child with a malabsorption syndrome who was put on a soy-free diet until age 3 years. The authors concluded that delaying introduction of cow milk by feeding infants soy formula until 9 months of age did not diminish the incidence of cow-milk intolerance.

Strengths/Weaknesses: The children in this study had a strong family history of allergy. Strengths include the random assignment to formula-feeding group and specified time that children were fed in that manner. There was long-term follow-up with repeated blood measures of immune status. The small sample size is a weakness. There was no breast-fed comparison group, and all infants were breast-fed for varying lengths of time before being randomized to formula group. Mothers could mix breast feeding and formula feeding, making effects of 1 food source impossible to determine. Statistical methods were not shown.

Utility (Adequacy) for CERHR Evaluation Process: This report is of no utility in the evaluation process.

Klemola et al., 2002 ***(554)*****,** supported by Turku University Hospital, the Social Insurance Institution, and Valio Ltd., evaluated 170 children with cow milk allergy for the development of allergic symptoms on soy formula or extensively hydrolyzed formula. The children were diagnosed with cow milk allergy at age 2–11 months using a double-blind placebo controlled food challenge. Children were randomly assigned by center and age to extensively hydrolyzed formula (n=90) or soy formula (n=80) until age 2 years. **[Both formulas were manufactured by Valio Ltd.]** Other sources of soy protein, egg, fish, citrus, peanuts, peas, tomatoes, strawberries, and chocolate were avoided for the first year. When the study formula was suspected of causing symptoms, a double-blind food challenge was performed

using both formulas, and the child was switched to the opposite study formula. If both formulas were believed to cause symptoms, the child was put on an amino acid formula. At the time of diagnosis of cow milk allergy and at ages 12 and 24 months, children underwent skin testing and specific IgE antibody measurement. Skin testing was performed with dried cow-milk formula, soy formula, the extensively hydrolyzed formula, a negative control, and a positive control (histamine). Statistical comparison of the incidence of adverse reactions to each study formula with 95% confidence intervals was performed using an exact binomial distribution. Chi-squared testing was used for categorical variables.

Parents suspected adverse reaction in 28% of subjects on soy formula and 11% of subjects on extensively hydrolyzed formula (RR 2.48 **[95% CI 1.28–5.12**]; *P*=0.006). Of the adverse reactions that were followed by a controlled food challenge, 10% were positive in the soy formula group and 2.2% were positive in the extensively hydrolyzed formula group (RR 4.50 **[95% CI 1.12–28.00]**, *P*=0.031). Some of the reactions during the challenge were characterized as doubtful and most reactions were not supported by IgE and skin test data. During the 2 year follow-up period, a similar number of children assigned to each formula had IgE antibodies to soy protein. Among children younger than 6 months who were assigned to soy formula, 40% were suspected by parents of adverse reactions. **[Data in 6-month-old children were not reported for the comparison formula.]** The authors concluded that more than 70% of infants with cow milk allergy tolerated soy formula and that IgE-mediated reactions to soy formula were unusual. They indicated that soy formula may be considered a first choice for cow milk intolerant children with the possible exception of children younger than 6 months.

Strengths/Weaknesses: Randomization to 2 different formulas and the adequate sample size are strengths of this study. Weaknesses are the lack of clear criteria for what constituted an adverse response and the failure of laboratory testing to confirm many of the presumed hypersensitivity reactions. In addition, because the study extended to age 2 years, there would have been different lengths of follow-up for the children from as little as 13 months to as much as 22 months.

Utility (Adequacy) for CERHR Evaluation Process: This study is of limited utility in the evaluation process.

Klemola et al., 2005 ***(555)*****,** funded by the Finnish Social Insurance Institution, Turku Hospital, and Valio, Ltd., performed a prospective, randomized study of an extensively hydrolyzed whey formula (PeptidiTutteli®; n=90 at study onset, n=76 after drop-outs) or soy formula (SoijaTutteli®; n=80 at study onset; n=72 after drop-outs) in infants with cow milk allergy. **[Both formulas were made by the corporate sponsor of the study.]** Infants were diagnosed with cow milk allergy based on double-blind food challenge at a mean age of 7 months (range 2–11 months). Infants were given no other source of soy protein during the first year, and other specific foods were avoided if skin testing suggested sensitivity. **[Skin testing is not reliable at this young age.]** Infants were examined at age 2, 3, and 4 years. Serum IgE levels were measured in infants at randomization, and at age 1, 2, and/or at 4 years **[not all children were evaluated at all time points]**. Parents were questioned at age 18 months, and 2, 3, and 4 years about food avoidance and adverse reactions to soy or peanuts. Chi-squared, Pearson correlations, and multivariate logistic regression was used to analyze the data.

Detectable soy IgE levels were numerically more prevalent in the soy formula-fed infants at all ages, but the difference from the hydrolysate-fed group was never statistically significant. At 4 years, the OR (95%

CI) for soy IgE positivity among soy formula-fed infants was 2.28 (0.90–5.76; *P*=0.082). No difference was detected between the groups in prevalence of peanut IgE positivity (OR 1.27, 95% CI 0.35–4.71; *P*=0.717). None of the 4-year-old children were reported to have had an immediate reaction to soy products, although 1 child in the soy formula-fed group had a delayed reaction (eczema). No difference was detected between the groups in the proportion of children with reported reactions to peanuts. The authors concluded that feeding soy formula to children with cow milk allergy may slightly increase the risk of sensitization at age 4, but that clinically important reactions to soy products were uncommon. They also concluded that feeding soy formula did not increase the risk of sensitization to peanuts.

Strengths/Weaknesses: Strengths include the randomized design with long term follow-up to four years of age and the biological measures of sensitization. However, the reasons for the 22 dropouts were not given. With respect to adverse reactions to peanuts, it unclear how many children in the sample were really exposed to peanut products; it appears that approximately one-third did not think they ever had been exposed. If cross-reactivity is the reason for the high correlation between IgE antibodies to soy and to peanuts, it is difficult to understand why it would be expected that use of soy formula would increase the risk of peanut-specific IgE.

Utility (Adequacy) for CERHR Evaluation Process: This paper is of limited utility in the evaluation process.

Koplin et al., 2008 ***(556)*** used data from the Melbourne Atopy Cohort Study (MACS) to evaluate whether soy consumption in infants increased the likelihood of developing peanut sensitization by 2 years of age. Support for the study was provided by Nestle Australia; Dairy Australia, Cooperate Research Center for Asthma; VicHealth; and the Ilhan Food Allergy Research Foundation. In addition, a number of the authors are recipients of National Health Medical Research Council Career Development or Australian Postgraduate Scholorship awards. The MACS involves 620 infants considered to be at higher risk of peanut sensitization based on a family history of atopic dermatitis, asthma, hay fever, or food allergy. Mothers of infants in the MACS were recruited during pregnancy between February 1990 and November 1994 through the Mercy Hospital Antenatal Clinic in Melbourne, Australia. Following the period of exclusive breastfeeding, infants were randomly assigned to receive soy formula (ProSobee, Mead Johnson), cow's milk-based formula (NAN, Nestle), or a partially hydrolyzed cow's milk-based formula (NAN HA, Nestle). **[The period of exclusive breastfeeding was not defined in Koplin et al., 2008, but a description of the Melbourne Atopy Cohort Study presented in Lowe et al., 2006** ***(557)*** **states that the median duration of exclusive breastfeeding was 18 weeks (interquartile range, 10-22 weeks). The Koplin et al., 2008 publication did not present information on whether a casein- or whey-hydrolysate was used.]** However, parents of infants in any of the 3 feeding groups were permitted to give their child soy formula or soy milk at any time ("parent-selected"). Detailed surveys to collect information on diet, including when foods were introduced and if they were tolerated, were administered every 4 weeks from birth to 15 months of age and again at 18 months and 2 years. Skin prick test to determine peanut, milk, or egg sensitization were given at 6 months, 1 year, and 2 years of age, however, only those children for which a skin prick test was available at 2 years were included in the current study. In addition, parent report was used to assess eczema or cow's milk allergy. Multiple logistic regression was used to calculate odds ratios and 95% CI adjusted for confounding factors.

Skin prick tests were available for 449 children at 2 years of age (n=145 in the soy-based formula

group, n=158 in the cow's milk-based formula group, n=146 in the partially hydrolyzed cow's milk-based formula group). The percentage of children in each feeding group who received the randomly allocated formula ranged from 84.8–86.7%. A sizable percentage of the children in all three groups received "parent selected" soy formula (22.8–35.9%) or soy milk (19–28.3%). In the unadjusted analyses, the authors reported that no significant association between consumption of the randomly allocated soy formula and peanut sensitization. However, they detected a significant association between parent-selected use of soy formula or soy milk and peanut sensitization (OR=2.02, 95% CI=1.04–3.92, *P*=0.039). Additional analyses indicated that parent-selected soy consumption was more likely to occur in children with indications of milk sensitization or allergy or when milk allergy was present in the child's mother or siblings. Peanut sensitization was more likely in children who had siblings with milk allergy or in children who were sensitized to milk or eggs by 6 months of age, had eczema by 2 years of age, or parent-reported cow's milk allergy. Sensitization to cow's milk at 6 months, parent-reported symptoms of cow's milk allergy, and history of milk allergy in siblings were considered potential confounding variables because they were associated with both soy consumption and peanut sensitization. After adjustment for these factors, the OR between peanut sensitization at 2 years and consumption of parent-selected soy formula or soy milk was no longer statistically significant (OR=1.34, 95% CI=0.64–2.79). The authors suggest that earlier reports of soy ingestion and peanut allergy may be explained by the selective introduction of soy to infants with a family or personal history of cow's milk allergy. In addition, they conclude that the avoidance of soy is not an effective strategy to prevent peanut allergy in infants of atopic families.

Strengths/Weaknesses: Self selection of changing formulas is a major confounding variable in interpreting the results of the study.

Utility (Adequacy) for CERHR Evaluation Process: The study is of no utility for the evaluation.

Lack et al., 2003 *(558)*, supported by the Medical Research Council, the Wellcome Trust, and several UK government agencies, used data from the Avon Longitudinal Study of Parents and Children to determine what factors, including soy intake, affected development of peanut allergy. From a large prospective cohort study of 13,971 preschool-aged children, 49 children with peanut allergy were identified and compared to controls using data retrospectively collected by masked telephone interview. One control group of 70 atopic children was randomly selected from those who were reported to have eczema or whose mothers had a history of eczema. A second control group consisted of 140 randomly selected children who did not have peanut allergy. The telephone survey of case and control parents was designed to collect additional information on maternal peanut consumption during pregnancy and lactation, family history of peanut allergy, and use of peanut oil-containing skin lotions and creams. Of the 49 children identified, 36 underwent skin testing and 29 tested positive for peanut allergy. There was a statistically significant association between soy product consumption and both peanut allergy and positive peanut challenge. Soy-milk or soy-formula consumption in the first 2 years occurred in 8.3% of the children in the total cohort, compared to 24.5% with peanut allergy and 34.8% with positive peanut challenge. In addition, 9 of the 10 children for whom consumption history was available were exposed to soy prior to peanut reaction. Step-wise logistic regression analysis, controlling for significant risk factors (rash over joints and in skin creases and oozing, crusted rash), confirmed that early soy consumption was an independent risk factor for peanut allergy (OR 2.61, 95% CI 1.31–5.20) and positive peanut challenge (OR 3.15, 95% CI 1.27–7.80).

The possibility that peanut allergy was associated with underlying cow-milk allergy rather than with soy product intake was examined, but cow-milk allergy was determined not to be a confounder. Only 4 of the 289 children in the cohort who were allergic to milk also had peanut allergy. The authors speculated that although no child in the study reacted to both peanuts and soy products, an immunologic co-reactivity between peanuts and soybeans could have caused cross-sensitization without clinical soy product allergy. Alternatively, the authors noted that an unidentified confounding factor could have been responsible for the observed relationship.

Strengths/Weaknesses: This nested case control study within a large prospective cohort study included 2 comparison groups, 1 "diseased" (atopic children) and 1 normal. It appears that soy-product feeding data were collected prospectively with respect to outcome. There were appropriate multivariate analyses. The reasons for soy product consumption in the first place could have confounded the association between soybean and peanut allergy. This possibility was likely addressed by the atopic control group, but the reported comparisons seem to have been conducted with both control groups combined.

Utility (Adequacy) for CERHR Evaluation Process: This study is of limited utility for the evaluation process.

May et al., 1982 ***(559)***, supported by the Public Health Service and the National Dairy Council, measured serum antibodies to 5 major milk proteins and a soy-protein isolate in 67 infants fed 1 of 2 cow-milk preparations, a soy product (EdiPro A), or a mixture of 73% cow milk/27% soy isolate from birth to 112 days of age. The infants were then switched to another product. Serum samples were obtained at 28, 112, 140, 168, and 196 days of age. Samples were also obtained from 7 infants who were breast-fed from birth to 112 days. Additional data were obtained from 60 older children up to 16 years of age from a separate food-sensitivity study in which subjects received unrestricted diets including cow milk. The serum antigen binding capacity (total antibody content) was determined using radiolabeled antigens. Data were analyzed using the Student *t* test. After 112 days, binding of soy protein in the serum of soy formula-fed infants was significantly less than binding of milk proteins by infants fed only cow milk. Feeding a soy product from birth resulted in a comparable or greater antibody response to subsequent cow-milk introduction than was seen in infants fed cow milk from birth. In light of this finding and a decreased antibody response to heat-treated milk, the authors suggest that heat-treated cow-milk formula rather than soy formula may be a preferred substitute for human milk.

Strengths/Weaknesses: There were 4 feeding groups with defined exposures, and there was some information about solid food intake. It is a weakness that the data were not originally collected for this study but rather were from different sites with different initial protocols. The group sizes were small.

Utility (Adequacy) for CERHR Evaluation Process: This study is of no utility for the evaluation process.

McDonald et al., 1984 ***(560)*****,** funding not indicated, conducted a study to evaluate immunologic mechanisms involved in food protein-induced entercolitis. Eighteen infants (14 male) presented with vomiting and diarrhea that continued after switching to soy formula and had stools containing mucus, blood, and leukocytes. Symptoms disappeared within 2–3 days of removing whole protein from the

diet. Class-specific antibodies to ovalbumin, soy, and cow milk were measured before food challenges to obtain baseline levels. Infants were then challenged with sequential oral 0.6 g protein/kg bw doses of egg white, soy formula, and skim milk. Forty-eight hours or 5 days were allowed for re-attainment of baseline values following either a negative or positive challenge, respectively.

Infants with positive challenges to soy formula and egg had significantly higher IgG anti-soybean antibody concentrations than those with negative responses. Similarly, children with positive challenges to soy formula and egg also had higher IgA food-antibody titers. IgM levels did not differ between positive and negative challenge responders. However, in 12 infants who were followed after a single soy formula challenge, IgM anti-soybean antibody was found to increase with a negative response and decrease with a positive response. The authors stated that these findings suggested altered immunologic responses to ingested antigens in infants having food protein-induced enterocolitis.

Strengths/Weaknesses: The report included a well defined group with confirmed disease and looked at a range of antibodies. It is a strength that baseline, challenge, and post-challenge values were included. Weaknesses include the small sample size, the unknown or poorly described feeding histories, and the broad subject age range.

Utility (Adequacy) for CERHR Evaluation Process: This study is of no utility in the evaluation process.

Miskelly et al., 1988 ***(561)***, supported by Wyeth Laboratories and the Welsh Scheme for the Development of Health and Social Research, compared the effects of feeding cow-milk or soy formula on the development of allergic symptoms in 487 infants at risk of allergic disease because of family history. Women randomized to the intervention group were advised not to give their infants cow milk and to restrict their own cow-milk intakes to a half-pint per day. They were supplied with a soy formula to supplement or replace breast feeding. Mothers in the control group were not given instructions on any diet restrictions, and most infants in this group received cow milk. Babies were examined for allergic symptoms at 3, 6, and 12 months by a physician who was unaware of the babies' formula types. Skin tests for milk, cod, egg, wheat flour, and soy antigens were performed at 6 and 12 months. Feeding diaries were kept by the mothers. The proportions of infants with symptoms were evaluated using a chi-squared test with Yates correction.

Mothers in the intervention group were less likely to breast feed than those in the control group. The authors suggested that the decreased breast feeding in the intervention group was due to the free supply of soy formula given to women in this group. The only significant symptomatic difference detected in the first year of life between the 2 groups was in the incidence of oral thrush (51% soy formula compared to 40% breast feeding, $P<0.05$). When the effects of breast feeding were evaluated, infants who were never breast-fed had a significantly higher incidence of wheezing and of diarrhea than infants who were breast-fed. There was also a numerically higher incidence of nasal discharge (not statistically significant). Skin testing showed that the only food antigen associated with eczema was egg, with positive tests occurring in 15% of babies with a history of eczema compared to 2% of babies without eczema ($P<0.001$). The authors concluded that breast feeding was highly advantageous and that their findings gave no support to the hypothesis that withholding cow milk reduces the risk of allergic disease.

Strengths/Weaknesses: Strengths include the large sample size and the homogeneous group of infants at risk of allergy due to family history. The intervention was a combination of breast- and soy formula-fed infants, which is an important weakness. There does not appear to have been a separate analysis for soy formula-fed infants, so lack of differences between groups could have been due to failure to compare subgroups of the intervention group to the control group.

Utility (Adequacy) for CERHR Evaluation Process: This report is of no utility in the evaluation process.

Moore et al., 1985 ***(562)*****,** supported by the UK Medical Research Council, conducted a study of the breast-fed infants of atopic parents. The study objective was to determine the effectiveness of breast feeding in the prevention of atopy. Women in an experimental group (n=250) were asked to breast feed their babies for the first 3 months, avoid giving them solids, and to use a soy formula if any extra food was necessary. Women in the control group (n=275) used a cow-milk formula. The infants were seen daily until their discharge from the hospital and then at 3, 6, and 12 months. The mothers kept feeding diaries during the first 3 months. At 3 months, detailed feeding histories were obtained from the mothers, and the infants were clinically examined. The proportion of infants having eczema at 3 months of age was related to the time they were introduced to cow milk, with more than twice as many of those who were given cow milk in the first 4 weeks developing eczema as those introduced to cow milk during weeks 5–8 **[level of significance not indicated].** Significant differences were found in the prevalence of eczema at 3 and 6 months between infants who had received cow milk in weeks 1–4 and those who had not. No significant differences were detected between groups at 12 months. Multivariate analysis identified breast feeding as a protective factor. There was no detectable advantage of using soy as opposed to cow-milk formula as an artificial food source.

Strengths/Weaknesses: Strengths include use of a homogeneous group with positive history of eczema or asthma. Women who agreed to participate could be randomly assigned to breast feeding for at least 3 months or to standard advice. The multivariate analysis and the initially large sample size are additional strengths. The study was, however, primarily focused on comparing breast feeding to cow-milk formula. It appears that few women actually fed their infants soy formula, and almost none used this method exclusively. Consequently, the sample was not adequate for a comparison of soy formula-fed infants to cow milk-formula or breast-fed infants.

Utility (Adequacy) for CERHR Evaluation Process: This report is of no utility in the evaluation process.

Mortimer, 1961 ***(563)***, funding not indicated, reported a case study of soybean anaphylaxis in a male child with asthma and eczema. The anaphylactic reaction occurred at 20 months of age. The child had previously been exposed to soy formula, and the family lived near a soy-processing facility. At age 3 years, the child was slowly re-introduced to soy milk to test the hypothesis that allergenicity would decrease with avoidance of antigen. No reaction was seen. The author concluded that soybean allergenicity should be re-examined to determine the potential for soy to approach cow milk as an allergen in light of increasing exposure in the general population.

Strengths/Weaknesses: This case report is described in detail, but it is still just a case report.

Utility (Adequacy) for CERHR Evaluation Process: This report is of no utility in the evaluation process.

Perkkio et al., 1981 ***(564)***, supported by the Finnish Foundation for Pediatric Research and the Paolo Foundation, conducted a morphometric and immunohistochemical study of jejunal biopsy specimens from 5 infants with soy allergy, all of whom also had cow-milk allergy. The biopsies were taken when soy was the only food source for the infants, and they were compared to biopsies taken before soy feeding and after soy had been eliminated from the diet. Biopsies were either processed for routine histology or examined by direct immunofluorescence or by the direct immunoperoxidase technique. In 4 infants, serial blood samples were taken for measurement of immunoglobulins, complement fractions, and soy antibodies. During soy feeding, biopsies of the 5 study patients showed partial or total villous atrophy associated with crypt hyperplasia. Inflammation of the lamina propria and the epithelium was seen and was similar both to that seen in intestinal cow-milk allergy with malabsorption and to that seen in celiac disease. There were also increased numbers of plasma cells and IgA- and IgM-containing cells in the lamina propria, as well as increased numbers of intraepithelial lymphocytes. Soy antibodies appeared and IgA also increased in the serum at the time of the reaction to soy. When soy feeding was stopped, the morphology of the jejunum improved and cell numbers were reduced to normal. The authors concluded that intestinal soy allergy was similar to intestinal cow-milk allergy and was readily reversible.

Strengths/Weaknesses: This report included careful clinical and histological descriptions of infants with gastrointestinal sensitivity to soy formula, including comparison of biopsies before and after soy feeding and demonstration of reversal of changes. It is a weakness that all infants had known previous cow-milk formula sensitivity as well.

Utility (Adequacy) for CERHR Evaluation Process: This report is of no utility in the evaluation process.

Odze et al., 1993 ***(565)*****,** funding not indicated, reported biopsy findings from 20 infants with allergic proctocolitis. The authors evaluated a possible association between numbers of eosinophils in colonic mucosal biopsy specimens and infant food source. The diagnosis of allergic proctocolitis was based on the presence of rectal bleeding in infants younger than 1 year who responded to a change of diet and in whom no other cause of rectal bleeding was found. Of the 20 infants, 7 had been fed human milk, 5 cow-milk formula, 5 soy formula, and 3 a combination. Cow milk-fed infants presented at a mean age of 21 days, soy formula-fed infants at 36 days, breast-fed infants at 85 days, and combination infants at 144 days. These differences were not significant. The signs and symptoms of proctocolitis were similar in the different feeding groups. No significant relationship was found between the number of eosinophils in the mucosa and infant diet.

Strengths/Weaknesses: A strength of this case series is the use of biopsies with quantification of eosinophils.

Utility (Adequacy) for CERHR Evaluation Process: This report is of no utility in the evaluation process.

Ostrom et al., 2002 ***(548)***, supported by the Ross Products Division, Abbott Laboratories, conducted a feeding trial to evaluate the immune status and morbidity of infants fed soy protein-isolate formulas

with and without added nucleotides. Infants of mothers who decided not to breast feed before enrollment were randomly assigned to receive soy formula (n=92) or soy formula with monomeric nucleotides added at 74 mg/L (n=94) exclusively, from enrollment to 4 months. Both of these formulas, based on Isomil®, contained nucleotides 300 mg/L prior to supplementation. A third non-randomized group of 81 infants received human milk exclusively for up to at least 2 months and then received cow-milk formula supplements (Similac® with Iron; total nucleotides, 7 mg/L). Recommended immunizations were administered at 2, 4, and 6 months according to the 1994 immunization schedule of the American Academy of Pediatrics. Immune status of the infants was assessed by comparing morbidity and antibody responses to specific immunizations. Blood samples were taken at 6, 7, and 12 months of age. Antigen-specific immune responses, immune cell populations, and serum IgG and IgA were measured. ANOVA and ANCOVA models with the Tukey-Kramer method were used to compare continuous data from the 3 groups, controlling for site. Proportions of children with diarrhea, otitis media, and antibiotic usage were analyzed using chi-squared test.

No significant differences were detected between the 3 groups at 6, 7, or 12 months of age in total serum IgG or IgA concentrations or in serum concentrations of antibodies against poliovirus (IgA), diphtheria (IgG), or tetanus (IgG). Although no significant differences were detected in response to *Hemophilus influenza* b between infants fed soy formula with or without added nucleotides, infants fed nucleotide-supplemented soy formula had significantly higher geometric mean levels of antibody to *Hemophilus influenza* b at 7 and 12 months than did infants fed human milk. Although diarrhea reported by parents did not differ by feeding group, physician-reported diarrhea was significantly less frequent in the human milk-fed group compared to both soy-formula groups. No feeding-group differences for otitis media or antibiotic usage were detected.

The authors concluded that infants fed soy-based formulas have normal immunoglobulin levels, normal responses to vaccine antigens, similar morbidity associated with otitis media, and thus normal immune development compared with breast-fed infants. They also concluded that nucleotide supplementation of soy formula provided no benefit other than a marginal benefit in response to *Hemophilus influenza* b immunization.

Strengths/Weaknesses: Strengths include random assignment of infants whose mothers did not choose breast feeding, randomization within site, blinding of assignment, and the block design accounting for infant sex. The sample size was relatively large and an appropriate repeated-measures analysis was used for for vaccine response data. Weaknesses include the varying lengths of time for which breast-fed infants were fed exclusively by that method and the lack of baseline immune function data prior to vaccination. There could have been selection bias in women who elected to breast feed.

Utility (Adequacy) for CERHR Evaluation Process: This study is of limited utility for the evaluation process.

Whitington, 1977 ***(566)***, support not indicated, presented case reports on 4 infants with soy-protein intolerance. The 4 infants developed diarrhea during the first month of life while on cow-milk formula. Switching to soy-based formula resulted in clinical deterioration. Responses to soy-challenge tests included diarrhea, vomiting, hypotension, lethargy, and fever. Switching to a diet free of soy or cow-milk protein was followed by recovery and weight gain.

Strengths/Weaknesses: This case series involves severe clinical courses in infants sensitive to soy formula. It is a weakness that all infants first demonstrated sensitivity to cow milk formula before responding poorly to soy formula.

Utility (Adequacy) for CERHR Evaluation Process: This report of no utility in the evaluation process.

Zoppi et al., 1979 ***(567)***, funding not indicated, evaluated full-term infants given soy formula for the first 4 months of life. Three different protein levels were used (2.5, 4.0, and 5.5 g/kg/day **[kg bw or kcal formula was not specified; assignment method was unclear]**). There were 39 infants in the study, 3 of whom were hospitalized for illness and were not included in the results. Diet was adjusted every 2 weeks based on infant body weight. Blood samples were taken early in the fifth study month for determination of hemoglobin, red blood cell count, protein electrophoresis, immunoglobulins, urea nitrogen, creatinine, glucose, electrolytes, acid-base status, and amino acids. Data were compared using Student *t* test.

No significant differences in gains in weight and length over the 4-month observation period were detected between groups. Hematologic parameters were within the normal range and no differences between groups were detected. Concentrations of urea nitrogen reflected the different nitrogen intakes. Serum protein concentration was described as slightly higher in the high-protein group, although not significantly different from the other 2 groups. Serum albumin and α- and β-globulins were similar between groups. γ-Globulins increased with increasing protein intake. An increase in immunoglobulin concentration with increasing protein level was also described; however, due to the large SDs, only differences in IgG were significant. The authors noted that the serum levels of γ-globulin and immunoglobulins in the infants on the high-protein diet were similar to those reported in a different study in infants fed less than half this amount of cow-milk protein. The authors also reported that morbidity was higher in the low-protein group than in the other 2 groups, with the low-, mid-, and high-protein groups having 3.18, 2.13, and 2.00 infections per infant, respectively. **[A breastfed infant fed ad libitum would get approximately 1.8 to 2g protein per kg per day. Even after adjusting for the differences between soy protein and that present in human milk and the need for additional amino acids and protein in soy formulas, the amount of protein fed is largely in excess.]**

Strengths/Weaknesses: It is a strength that blood measures after 4 months of feeding were associated with the level of soy protein. Infections were not well described, and no significance testing was used. The sample size was small.

Utility (Adequacy) for CERHR Evaluation Process: This study is of no utility in the evaluation process.

Zoppi et al., 1982 ***(568)*****,** supported by "CNR and MPI grants," compared immunocompetence in infants fed formulas containing either cow-milk protein or soy protein. Infants were assigned to 1 of 4 feeding groups, as shown in **Table 88** **[method of assignment unclear; previous study cited]**. Two different protein concentrations were obtained for each formula by diluting with water and adding dextromaltose to maintain caloric content. The infants received their assigned formulas from birth to 4.5 months and were examined every 2 weeks for weight, length, and illness (chiefly infection). After 4.5 months, blood samples were evaluated for hemoglobin, hematocrit, white blood cell count, protein electrophoresis, urea nitrogen, creatinine, glucose, electrolytes, iron, transferrin, cholesterol, and triglycerides. Immunoglobulin, complement, and amino acid determinations were performed. Tests

Table 88. Energy Composition of Reconstituted Formulas (Zoppi et al., 1982)

Protein, g/kg/day	*n*	*Protein Concentration, g/100 mL*	*% kcal from Protein*	*Calories, kcal/100 mL (kJ)*	*Protein:Calorie Ratio, g protein/100 calories*
Cow-milk formula					
2.0	7	1.6	8.6	74.2 (310.4)	2.2
4.0	7	3.0	15.9	75.4 (315.5)	4.0
Soy formula					
2.0	13	1.6	8.6	74.2 (310.4)	2.2
5.0	14	3.5	21.0	70.5 (295.0)	5.3

[Per kg assumed to be per kg bw].
From Zoppi et al., 1982 *(568)*.

of immune function were performed on isolated monocytes and included rosette formation to sheep erythrocytes, estimation of surface immunoglobulins, and mitogen response to phytohemagglutinin, pokeweed mitogen, concanavalin A, and *Staphylococcus protein A*. Data were analyzed using the 2-sample *t* test or the Beherens-Fisher test and multivariate linear discriminant analysis.

No differences in head circumference and mean gains in weight and length were detected between the groups. There were more episodes of infection in infants fed the low-protein soy formula (3.4 episodes per infant) than in infants in the other groups (1.8–2.6 episodes per infant **[significance levels not given]**). Hematologic parameters fell within normal ranges for all groups; although in comparison to other groups, the authors noted a significantly lower hematocrit in the low-protein soy group, a higher white blood cell count in the high-protein soy group, and higher iron concentration (with lower transferrin values) in infants fed either soy formula. Serum cholesterol and triglycerides were lower in the 2 soy groups**. [Levels of significance were not given for these comparisons.]**

Total serum protein, albumin, and β-globulin, and γ-globulin levels were described as higher in the cow-milk than soy groups, although the differences were not significant. Serum gamma-globulins, IgA, IgG, and IgM were significantly lower in the low-protein soy group than in the other groups. Complement C3 levels were lower in soy formula-fed infants than in those receiving cow milk. Complement C1 and C3 levels were lower in the 2 low-protein groups compared to the 2 high-protein groups. No statistically significant differences were detected between groups in monocyte function, although the authors reported a "tendency" for T cells from infants in high-protein groups to show a greater response to mitogens. Discriminant analysis incorporating γ-globulins, transferrin, complement (C4 and C1), activated rosette formation, and mitogenic response showed clear separation of groups by protein amount and source (cow milk or soy). Using only T-cell function parameters, a separation between groups was evident with a comparison of low-protein soy and high-protein cow-milk groups and, to a lesser extent, between low- and high-protein cow-milk groups. The study authors concluded that soy protein was of lower nutritional value than cow-milk protein, and that low-protein formulas were suboptimal with respect to immune function. **[It is unclear on what basis the authors made this conclusion.]**

Strengths/Weaknesses: The strengths and weaknesses of this study are similar to those of the previous study *(567)*.

Utility (Adequacy) for CERHR Evaluation Process: This study is of no utility in the evaluation process.

Zoppi et al., 1983 ***(569)***, funding not indicated, studied the relationship between formula type and antibody response to vaccination in 62 healthy infants born at term. The study included 27 infants whose mothers decided to breast feed. Other study infants were randomly assigned to a high-protein cow-milk formula (4.4 g/kg /day; n=7), a low-protein cow-milk formula (1.6 g/kg/day; n=9), an adapted cow-milk formula with a casein/albumin ratio of 40:60 (protein 1.8 g/kg/day; n=10), or a soy formula (protein 4.6 g/kg/day; n=9). Infants received their assigned formula exclusively until 5 months of age when they were all switched to the same diet (with ~3.5 g/kg/day of protein) and followed to 1 year of age. **[The Expert Panel assumes the per kg designations are per kg bw.]**

Weight and length were measured every 2 weeks. Infants were vaccinated against poliovirus, diphtheria, tetanus, and pertussis at 2 and 4 months of age. Blood was sampled at 5 and 8 months of age and analyzed for antibodies, immunoglobulins, hemoglobin, hematocrit, white blood cell count, protein electrophoresis, urea nitrogen, creatinine, glucose, electrolytes, iron, transferrin, cholesterol, and triglycerides. Data were evaluated using ANOVA **[post hoc tests not designated except as orthogonal]**.

All groups had normal hematologic variables, blood urea nitrogen, amino acid levels, serum protein, immunoglobulin, zinc, transferrin, and mean gains in weight, height, and head circumference. Groups differed in antibody response to vaccination. Breast-fed infants and infants fed the high-protein cow-milk formula had protective antibody levels at age 5 and 8 months. Antibody levels in infants fed the adapted formula were significantly higher at 5 months but lower at 8 months than those of breast-fed infants. In infants fed the low-protein cow-milk or soy formulas, antibody levels were significantly lower than in breast-fed infants. Infants fed soy formula had significantly lower levels of antibodies to tetanus, diphtheria, and pertussis than did the breast-fed group or the high-protein cow-milk group. However, infants fed soy, low-protein cow-milk, and adapted cow-milk formulas had significantly higher levels of antibodies to poliovirus type 2 than did breast-fed infants. The mean number of episodes of infection per infant was highest in the soy formula-fed group (2.9), compared to breast-fed (0.5), high-protein cow milk (1.6), low-protein cow milk (2.7), and adjusted formula (2.4) **[statistical significance not reported]**. The authors concluded that soy formula-fed infants had an impairment of antibody response to common viral and bacterial vaccinations, with the exception of poliovirus type 2. They suggested that recommended protein intakes should be re-assessed to take into account both the quality and quantity of dietary protein. They recommended an animal protein intake of 3 g/kg bw/day and concluded that vegetable protein should not be given to infants during the first months of life.

Strengths/Weaknesses: Strengths included a measure of immune function in infants, vaccination response, and the use of 4 feeding groups with random assignment after breast feeding-preference selection. Weaknesses included the small numbers, although significant differences were still identified on some measures, the possible selection bias for breast-fed mothers, and the lack of demonstrated clinical significance of the observed differences in immune response. It is difficult to understand the relevance of the number of infections related to immune response because of the lack of detail regarding the infection type. After 5 months, diet is unknown in the participants.

Utility (Adequacy) for CERHR Evaluation Process: This study is of no utility for the evaluation process.

3.2.1.4 Thyroid Function

Chorazy et al., 1995 ***(570)***, support not indicated, presented a case report of a child with congenital hypothyroidism who was placed on soy formula at birth due to parental concern about a family history of milk allergy. The child's thyroid-stimulating hormone could not be suppressed into the normal range with oral thyroxine supplementation until cow-milk formula was substituted for soy formula. Stool frequency decreased from 5–7/day on soy formula to 2–3/day on cow milk formula. The authors believed this report and a similar report from 1965 provided evidence that soy formula interferes with thyroxine absorption from the intestine through fecal wastage. They noted that although iodine supplementation of formulas and a reduction in the fiber content of soy formulas decreased the risk of thyroxine malabsorption, formula intolerance might have led to an increase in stool frequency and a risk of fecal wasting of thyroxine in their patient.

Strengths/Weaknesses: This case involves a special population of infants with congenital hypothyroidism. The case report format is a weakness.

Utility (Adequacy) for CERHR Evaluation Process: This report is of limited utility for the evaluation process.

Conrad et al., 2004 ***(571)***, funded by the Children's Memorial Institute for Education and Research, evaluated the hypothesis that feeding soy formula to infants with congenital hypothyroidism (CH) leads to prolonged TSH increase when compared to infants fed non-soy formula. Medical charts, from 1990 to 1998, of infants with congenital hypothyroidism were reviewed for clinical diagnosis, date of treatment initiation, T4 and TSH levels, levothyroxine dose, weight, length, and diet information during the first year of life. Subjects were assigned to the soy formula group (n=8) or non-soy formula group (n=70). Based on standards from the National Center for Health Statistics, each subject was assigned a percentile, z score, and percent median for height, weight, and weight for height. The etiology of hypothyroidism was determined by thyroid scan. Statistical analyses were conducted using the Wilcoxon rank sum test, Fisher's exact test, test of fixed effects, and Wilcoxon test for two groups of survival data were performed using SAS software.

Among the 78 subjects, 71% had thyroid dysgenesis and 17% had dyshormonogenesis. The remainder of subjects had a transient condition, Down syndrome, or had no thyroid scan. There was no difference in TSH and total T4 levels in the two groups at the initiation of levothyroxine treatment. There was a significant difference in the TSH levels at the first determination (approximately 50 days of age) following initiation of treatment (soy group median=42.6 mU/l, non-soy group median=6.6 mU/l, P=<0.01). The time required to reach a TSH level <10 mU/l also differed significantly between the two groups. For the soy group the median time required was 150 days while the non-soy group required a median of only 40 days (P=0.02). At 4 months of age, 62.5% of the soy group had high TSH levels while only 17% of the non-soy group had high TSH (P=0.01). This difference persisted throughout the first year of life. Overall, the soy group had higher TSH levels and a slower descent in these levels than the non-soy group. There was no difference in total T4 between the two groups. Also, there was no significant difference between the two groups with regard to weight, height, or weight for height. The authors speculate that the high and prolonged levels of TSH in the soy group may be due to malabsorption and increased fecal loss of levothyroxine.

Authors' conclusions: Infants fed soy formula had prolonged increase of TSH when compared to infants fed non-soy formula. These infants need close monitoring of free thyroxine and TSH measurements, and they may need increased levothyroxine doses to achieve normal thyroid function tests.

Strength/Weaknesses: This retrospective study's strengths included the availability from medical records of both pre- and post treatment measures of TSH and T4 as well as levothyroxine dose in the first year of life among children who had a diagnosis of congenital hypothyroidism. The study's weaknesses are that the exposure to soy formula was classified solely on the basis of a notation in the medical record that soy formula was used or not. There is no information on the reasons for soy formula use, the duration of formula use, and/or the timing of introduction of other foods. With such a small number of infants in the "soy diet" group, there may have been insufficient power to detect some differences at baseline, e.g., there was wide variability in baseline TSH levels pre-treatment. It is also not explained why dose of levothyroxine did not differ between groups over the first year of life, despite the fact that TSH levels remained high in more than half of the soy treated group. Finally, the outcome measure of growth was collected at the latest time point available in the medical record, so it is unclear how age of last growth measurement differed between groups. In addition, there is no measure of neurodevelopment - the most critical outcome for congenital hypothyroidism. For these reasons, it is difficult to interpret the results of this study.

Utility (Adequacy) for CERHR Evaluation Process: This study is of limited utility in the CERHR evaluation process.

Jabbar et al., 1997 ***(572)***, funding not indicated, reported 3 infants with congenital hypothyroidism who experienced apparent malabsorption of thyroxine while on soy formula. Two of the infants were found on laboratory monitoring to have elevated thyroxine levels after they had been switched from soy to cow-milk formula within the first 2 months of life. The thyroxine levels normalized with the thyroid replacement dose was decreased. The third infant did not suppress thyroid-stimulating hormone levels on thyroxine replacement doses as high as 19 µg/kg bw/day. Switching from soy formula to cow-milk formula resulted in normalization of thyroid-stimulating hormone with an administered thyroxine dose of 14 µg/kg bw/day. The authors concluded that soy formula could result in decreased absorption of administered thyroxine and decreased enterohepatic reabsorption of thyroxine with consequent fecal wastage.

Strengths/Weaknesses: These cases involve a special population of infants with congenital hypothyroidism. The case series format is a weakness.

Utility (Adequacy) for CERHR Evaluation Process: This report is of limited utility for the evaluation process.

Shepard et al., 1960 ***(573)***, supported by the Platex Park Research Institution, reported 3 children with goiter related to the feeding of soy formula. The infants were clinically euthyroid but had low-normal levels of protein-bound iodine and high ^{131}I uptakes. When soy formula was discontinued, ^{131}I uptake returned to normal. In 2 of the cases, the goiter decreased in size or disappeared with the discontinuation of soy formula. Reduction in goiter size occurred in the third case when iodine was added to the diet and the brand of soy formula was changed. The authors concluded that lack of iodine was the cause of the soy formula-associated goiter.

Strengths/Weaknesses: These cases involve a special population of infants with congenital hypothyroidism. The case series format is a weakness so is a lack of information on age, actual free T4 levels.

Utility (Adequacy) for CERHR Evaluation Process: This report is of limited utility for the evaluation process.

3.2.1.5 Reproductive Endpoints

Bernbaum et al., 2008 *(574)*, supported by the NIH, conducted a pilot study to characterize breast and genital anatomy, milk secretion, and vaginal cytology during the first 6 months of life in male and female infants. The authors were initially interested in assessing whether soy formula use prolonged physiologic estrogenization in newborns but they discovered that the natural history of breast and genital development was too poorly described to permit such a study. The specific purposes of the pilot study were to (1) examine the methods used to characterize breast and genital development in infants, and (2) assess which endpoints might exhibit declines during infancy, a pattern consistent with the response to the withdrawl of maternal estrogen.

A total of 88 physical exams from 72 children (44 examinations of 35 girls and 44 examinations of 37 boys) were conducted at the Children's Hospital of Philadelphia and affiliated clinics during 2004-2005. The total number of exams per child ranged from 1 to 4. The pilot study included 2 boys and 2 girls in each of seven age intervals (< 2 days; 1, 2, and 3 weeks; 1, 3, and 6 months) and three feeding regimens (breastmilk, cow milk-based formula, or soy formula). Some flexibility in feeding regimen was allowed immediatly after birth and in older infants because the primary purpose of the study was for methods development and not to test hypotheses related to feeding regimen. The physical exams were adapted from the New Ballard score and included assessment of breast bud diameter, palpability of breast adipose tissue, milk secretion, vaginal antatomy, vaginal discharge, vaginal wall cell cytology, and scrotal and testicular anatomy. Data analysis included descriptive statistics, paired t test to examine left-right differences in paired orggans, and tests for age trends.

Not all of the endpoints showed a developmental pattern consistent with a response to withdrawal of maternal estrogens. Breast bud diameter was highest at birth and then regressed in older infants. Genital development did not vary as a function of age. Milk secretion and withdrawal bleeding did not occur often. The maturation index of vaginal wall cells was maximal early in life and did not appear to differ based on feeding regimen in girls younger than 30 days. However, after that point the maturation index in female infants fed soy formula was higher compared to girls fed breastmilk or cow milk-based formula. The authors concluded that breast tissue and vaginal cytology appeared to be most responsive to the withdrawl of maternal estrogen. The pilot study was considered too small to draw reliable conclusions about the impact of feeding regimen on breast and genital development.

Strength/Weaknesses: Mixed cross-sectional design rather than randomized intervention; nonetheless, a reasonable design. Sample size very small (e.g., once gender is considered); study is likely to be underpowered to detect any meaningful associations. The original longitudinal study design required four measurements, but a great majority of the subjects had only one measurement. The study did not observe significant growth of the testes during the first year, further showing the lacking statistical power and inaccurate method of measurement. Hormonal data would add value to the study. Essentially

a pilot study to demonstrate feasibility and assess potential for any effect of exposure to breast milk, or cow-based or soy-based formulae.

Utility (Adequacy) for CERHR Evaluation Process: This study is of no utility to address primary areas of interest. However, it is useful in establishing validity and feasibility of study design and for indentifying an area for future research.

Boucher et al., 2008 ***(575)***, supported by the Canadian Breast Cancer Reasearch Alliance and the Canadian Breast Cancer Foundation Ontario Chapter, examined the relationship between infant soy formula consumption and breast cancer risk in a population-based case control study. Questionnaires were mailed to 4,109 registry-identified women aged 25-74 diagnosed with breast cancer between June 2002 and April 2003 and 4098 controls matched within 5-year age groups. Subjects were born between 1930 and 1977. The questionanaire included a request for consent to establish maternal contact to find out what study participants were fed during the ages of birth to 4 months and 5-12 months. Feeding options were: breast milk only, cow's milk formula only, soy formula only, breastmilk and cow's milk formula, breast milk and soy formula; cow's milk and soy formula, and "other." Responses related to feeding options during infancy were recieved from 372/418 mother's of women with breast cancer (cases) and 356/437 mothers of women without breast cancer (controls). Odds ratios were adjusted for a number of factors including age group, contraceptive use, postmenopausal estrogen replacement therapy use, breast cancer in 1st- or 2nd- degree relative, or history of benign breast disease. **[The specific factors adjusted for in the first 4 month and 5-12 month analyses overlapped but were not identical]**.

Although the point estimates of the odds ratios were less than unity, the variability of these estimates indicate that feeding soy formula was not statistically significantly associated with developing breast cancer (soy formula only during first 4 months of life: OR=0.42, 95% CI=0.13–1.40; soy formula only during 5-12 months of age: OR=0.59, 95% CI=0.18–1.90). The authors noted a number of limitations to the study, including low statistical power due to low soy formula use and the relatively few mothers recruited. Also, soy forumula use may have been greater in younger subjects. The authors considered recall and response bias unlikely in the responses from mothers of study participants.

Strengths/Weaknesses: The population-based case control design is sound. Some potential for recall bias but this is probably modest as the information was obtained from the mothers rather than subjects. Study population is of reasonable size but the ages of the population cover multiple decades and the role of all established risk factors (other than HRT, family history, prior benign disease) may (or may not) have been adequately considered. Number of variables and the limited number of responding mothers probably reduces power substantially. Not clear if this is a validated instrument for the population. Likely an underlying trend for prevalence in the use of different formulations over time; these could affect the ability to interpret exposures. Furthermore, not all soy or cow milk formulations may have been constant over time and other factors in the formulations could affect outcomes.

Utility (Adequacy) for CERHR Evaluation Process: This study is of limited utility in supporting further evaluation.

Freni-Titulaer et al., 1986 ***(576)***, funding not indicated, conducted a case-control study to evaluate factors associated with a 3-fold increase in the number of reported patients with premature thelarche

between 1978 and 1981 in Puerto Rico. Potential cases included girls between the ages of 6 months and 8 years with palpable breast tissue at least 1.5 cm in diameter at the time of diagnosis of premature thelarche in the absence of other evidence of premature sexual development. Of the 552 potentially eligible subjects diagnosed between 1978 and 1982 with premature thelarche, 397 parents returned consent forms, and 130 representative case subjects were systematically selected for the study. An age-matched control subject was selected for each case subject, including where possible, a friend of the case. Parents were interviewed to determine family history and possible exposures. Data were analyzed by the computation of OR with 95% CI using a matched-pairs analysis followed by step-wise logistic regression on all the variables. Multivariate analysis was used to control for confounding factors.

Consumption of soy formula was found to be associated with premature thelarche in the univariate analysis (OR 2.2, 95% CI 1.0–5.2, *P*=0.05 based on 22 exposed cases and 10 exposed controls). A maternal history of ovarian cysts was also significantly associated with premature thelarche on univariate analysis (OR 3.8, 95% CI 1.5–11.5, *P*=0.002). Multivariate analysis showed no significant associations overall. When the analysis was restricted to girls with onset of thelarche before age 2 years, consumption of soy formula remained significantly associated in multivariate analysis (**Table 89**). The study authors noted that in more than 50% of the subjects, there was no exposure to any of the risk factors for which statistical associations were found. They suggested that premature thelarche resulted from a variety of environmental and familial factors.

Table 89. Factors Associated with Thelarche Prior to 2 Years of Age on Multivariate Analysis (Freni-Titulaer, 1986)

Factor	*OR (95% CI)*	*P-value (two-sided)*
Soy formula	2.7 (1.1– 6.8)	0.029
Maternal ovarian cysts	6.8 (1.4–33.0)	0.017
Chicken consumption	4.9 (1.1–21.9)	0.035
Corn consumption	0.2 (0.0–0.9)	0.039

From Freni-Titulaer, 1986 *(576)*.

Strengths/Weaknesses: The case-control study design is a strength. Other strengths are the use of age-matched controls, the appropriate matched and multivariate analyses, and the reasonable sample size. Weaknesses include the broad age range of the girls with varying lengths of delay in collecting retrospective history and the use of friend controls in some cases but not in others. There could have been recall bias; for example, factors associated with thelarche prior to age 2 (in multivariate analyses) should have controlled for the age at which the maternal interview was conducted. Other potential confounders were not addressed, and the reasons why soy formula was used initially could have been a confounding association. Strengths include the sample size, population studied and the general design seems sound. The accuracy of maternal recall is likely to have been good for soy formula consumption. The strengths render the study useful for further evaluation.

Utility (Adequacy) for CERHR Evaluation Process: This report is of limited utility for the evaluation process.

Giampietro et al., 2004 ***(577)***, funding not indicated, conducted a retrospective study to determine the hormonal and metabolic effects of long-term feeding of soy formula in children. The study population

consisted of 48 children age 7–96 months who had been fed soy formula exclusively for at least 6 months. Of these children, 30 were given soy formula for documented cow-milk allergy and 18 for family history of allergy. The 48 children were divided into 3 age groups, 7–24 months (n=20), 25–72 months (n=20), and 73–96 months (n=8). An additional 18 healthy children aged 12–96 months who had not received soy formula during the first months of life were enrolled as a control group. The children were evaluated for height, weight, presence of breast budding, testicular size, presence of pubic/axillary hair, signs of precocious puberty in girls, and the appearance of gynecomastia in boys. Also studied were radiologic and biochemical markers of increased bone metabolism, including bone age, urinary deoxypyridoline, calcium, creatinine, and phosphate and serum levels of bone alkaline phosphatase, osteocalcin, 17β-estradiol, and parathyroid hormone. Univariate analyses were performed on the data, using the Fisher exact test and chi-squared test for discrete variables and the Mann-Whitney test for continuous nonparametric variables.

Height and weight were in the normal range for all children, and no differences were detected between soy formula-fed and control groups. No signs of precocious puberty in girls or of gynecomastia in boys were found. All 17β-estradiol concentrations were below the method detection limit of 20 pg/mL. No significant differences were detected in serum or urinary measurements, except that soy formula-fed infants had significantly lower urinary calcium and significant higher urinary phosphate in children 7–24 months old compared to control children.

The authors concluded that long-term feeding of soy formula in infants did not produce estrogen-like hormonal effects. They suggested that their findings confirm the theory that phytoestrogens have a low affinity for ERs and therefore produce only weak biologic effects. The authors suggested that the isolated differences in urinary calcium and phosphate should be confirmed in larger studies before conclusions are drawn.

Strengths/Weaknesses: It is a strength of this study that exclusive feeding of soy formula was apparently documented for at least 6 months in duration in the exposed group. Use of a comparison group of unexposed infants in the first months of life and multiple measures of possible hormonal effects are other strengths. Weaknesses include the retrospective enrollment of children, in some cases many years after infancy, wide age range at follow-up with very small sample sizes in each age stratum, particularly among controls, and the lack of clarity on how feeding patterns in early life were validated. The feeding practices in the comparison group were not well described. There were no multivariate analyses conducted or control for potential confounders.

Utility (Adequacy) for CERHR Evaluation Process: This report is of no utility for the evaluation process.

Gilchrist et al., 2009 ***(578)***, supported by USDA, examined differences in hormone-sensitive organ size at 4 months in infants who were fed soy formula (SF) (n=39, 19 males and 20 females), milk formula (MF) (n=41, 18 males and 23 females), or breast milk (BF) (n=40, 20 males and 20 females). Participants were drawn from an ongoing, longitudinal cohort (the Beginnings Study). Mothers had healthy pregnancies with no diagnoses or medications thought to affect infant growth or development, were non-smokers, and reported no use of alcohol, soy products or other estrogenic compounds during pregnancy and/or lactation. Infants were full-term (>38 weeks), had appropriate for-gestational age birth weight (6-9

pounds), and had no medical diagnoses or medications thought to affect growth or development. Parents chose the feeding method (BF, MF, or SF). All BF infants were exclusively fed breast milk the entire study time. 54% of the MF infants were exclusively fed MF from birth, 41% switched from BF to MF within 4 weeks, and 5% switched between 4 and 8 weeks. 23% of SF infants were exclusively fed SF from birth, 45% were switched to exclusive SF feeding within 4 weeks, and 32% were switched to SF between 4 and 8 weeks. At 4 months, anthropometric measures (weight, length, and head circumference) were assessed using standardized methods, and body composition was assessed by air displacement phlethymography. Breast buds, uterus, ovaries, prostate and testicular volumes were measured by ultrasonography. The Student' *t* test was used to test differences between MF-fed and BF-fed infants, SF-fed and MF-fed infants, and BF-fed and SF-fed infants. **[There was no adjustment for multiple pairwise comparisons.]**

Length of gestation (in weeks) was significantly greater in BF-fed girls and in SF-fed girls compared to MF-fed girls (P=0.03 and P=0.01, respectively). Weight (in kg) at 4 months of age for boys was significantly higher in MF-fed infants compared to SF-fed infants (P=0.04).

Among females no differences were observed in breast bud or uterine volume by feeding group. MF-fed infants had greater mean ovarian volume than BF-fed infants ($P\leq$0.04) and SF-fed infants ($P\leq$0.09). MF-fed and SF-fed infants also had a greater number of ovarian cysts per ovary than BF-fed infants ($P<$0.01) **[Interpretation of the ovarian cysts findings is based on a unit of measure of ovaries rather than per subject**.]

Among males, no differences were observed in prostate or breast bud volumes by feeding group. No differences were observed in mean testicular volume between SF-fed and MF-fed males, but both formula-fed groups had significantly lower volumes than BF-fed infants ($P\leq$0.04 for MF-fed and $P\leq$0.03 for SF-fed).

Strengths/Weaknesses: Comparison of human breast milk with soy and milk formulae is relevant. The study measured the effects of exposures on several biological endpoints that are directly relevant to the primary goals of this CERHR project. Mothers reported no use of soy or other estrogenic compounds during pregnancy or lactation, controlling for exposures that could have occurred *in utero*. However, cross-over among feeding sources commonly occurred, and many infants in each group were fed from more than one source. Thus, the level of exposure is not clear and the data are difficult to interpret (e.g., the study is open to potential confounding due to interactions among different feeding sources leading to possible overestimates or underestimates of effects). With relatively small numbers of each gender in each group, and cross-over common for the formula-based sources, only 23% of soy formula (n=9) and 54% of the milk formula (n=22) were fed their respective formula exclusively from birth. In addition, the primary endpoints were not defined a priori. The study is likely underpowered for meaningful analysis of the effects of soy formula on the developing reproductive organs.

Utility (Adequacy) for CERHR Evaluation Process: This study is of no utility to address primary areas of interest. However, it is useful in establishing validity and feasibility of study design and for identifying an area for future research. See discussion in "**Utility of Data," Section 3.5**.

Strom et al., 2001 ***(32)***, supported by Ross Products, Nestlé, and Mead Johnson, compared endocrine and reproductive outcomes in adults who had been fed soy or cow-milk formula during infancy.

Subjects were identified from a group of 952 individuals (248 fed soy formula and 563 fed cow-milk formula) who participated in controlled non-randomized infant feeding studies conducted at the University of Iowa between 1965 and 1978. In the feeding study, infants were enrolled before 9 days of age and fed either a cow-milk or soy formula through 16 weeks of age. The current study was conducted from March through August of 1999. Subjects included 248 individuals (120 male, 128 female) fed soy formula and 563 individuals (295 males and 268 females) fed cow-milk formula. Most subjects were Caucasian and were between 20 and 34 years of age. Information was obtained by structured and standardized telephone interviews with study participants. Men and women were asked about adult heights and weights, sexual maturation, and education level. Women were also questioned about menstrual cycles (cycle length, menstrual duration, flow, and pain) and reproductive outcomes (number of pregnancies, deliveries, and complications). Men were also asked about pregnancy outcomes in partners. Additional questions included congenital malformations in offspring, hormonal disorders, and homosexual orientation in both sexes, and testicular cancer in men. These outcomes were considered secondary and were expected to occur too infrequently to provide definitive data. Ordinal variables were analyzed by chi-squared or Fisher exact tests, and unadjusted and adjusted relative risks were calculated. Continuous variables were analyzed by *t* test or Wilcoxon rank sum test followed by linear regression. Confounding factors considered in the analysis included birth weight, age, usual body mass, parental weight and height, hormone disorders, smoking, alcohol intake, soy-food consumption, vegetarian diet, herbal supplement use, recreational drug use, physical activity, sexually transmitted diseases, and use of hormonal birth-control methods.

No significant differences were detected in adult height and weight, body mass index, or sexual maturation in men and women. Duration of menstrual bleeding was slightly longer in women from the soy-formula group (adjusted mean difference=0.37 days, 95% CI=0.06–0.68), but bleeding was not heavier. Borderline significance was obtained for the number of subjects experiencing discomfort during menstruation in the soy-formula group (unadjusted risk ratio (RR)=1.77, 95% CI=1.04–3.00 for extreme versus no or mild discomfort; adjusted RR was not calculable). No significant differences were identified for pregnancy outcomes in women. **[No reproductive parameters were reported for male subjects with the exception of sexual maturation. Although men were questioned about pregnancy outcomes in partners, the results were not reported.]** No significant effects were observed for secondary outcomes (i.e., cancer, reproductive organ disorders, hormonal disorders, sexual orientation, or defects in offspring **[data were not shown]**). With adjustment for multiple comparisons, the differences that had been identified were no longer statistically significant (more than 30 different endpoints were evaluated).

The study authors concluded that "Exposure to soy formula does not appear to lead to different general health or reproductive outcomes than exposure to cow-milk formula. Although the few positive findings should be explored in future studies, our findings are reassuring about the safety of infant soy formula."

In a letter to the editor, Goldman *et al.*, 2001 *(579)* discussed several perceived limitations of the Strom *et al.*, 2001 *(32)* study:

- Failure to mention in study abstract a higher use of asthma and allergy medications in subjects fed soy-based formula (P=0.08 in males and P=0.047 in females),
- Disregarding the slightly increased duration of menstrual cycle and greater menstrual discomfort

findings in soy formula-fed women, when those findings could indicate endometriosis or uterine fibroids, theoretical outcomes of early-life estrogen exposure,

- Inadequate assessment of female fertility by not considering time to pregnancy and use of fertility technologies and only asking subjects about subjective outcomes,
- Lack of reporting of male fertility outcomes or semen quality,
- Lack of assessment of cancer risk, and
- Lack of consideration of exposure to estrogenic compounds in various soy-based formulas.

In response to this letter, Strom *et al.*, 2001 provided incidences for thyroid disease (a possible autoimmune disease), endometriosis, uterine fibroids, low sperm count, and cancer in subjects fed soy versus cow-milk formula (**Table 90**). They noted the low number of subjects affected.

Table 90. Additional Findings in Subjects from the Strom et al., 2001 Study

Outcome	*Females, n (%)*			*Males, n (%)*		
	Soy (n=128)	*Cow Milk (n=268)*	*P*	*Soy (n=120)*	*Cow Milk (n=295)*	*P*
Thyroid disease[a]	5 (3.9)	6 (2.2)	0.34	1 (0.8)	0 (0.0)	0.29
Endometriosis	0 (0.0)	6 (2.2)	0.18	N/A	N/A	N/A
Uterine fibroids	0 (0.0)	2 (0.8)	> 0.99	N/A	N/A	N/A
Low sperm count	N/A	N/A	N/A	1 (0.8)	1 (0.3)	0.50
Cancer[b]	4 (3.1)	3 (1.1)	0.22	0 (0)	1 (0.3)	> 0.99

[a] Mainly hypothyroidism.
[b] Cervical in females, testicular in males.
N/A=Not applicable.
From the Strom et al., response to Goldman et al., 2001 *(579)*.

Strengths/Weaknesses: Strengths of the study include the relatively large sample size the prospective collection of feeding information, and the appropriate statistical analyses. Feeding assignment was, however, not randomized, soy-formula exposure was short-term (0–16 weeks of age), and information on subsequent feeding other than current vegetarian diet was not collected. Sample sizes for several endpoints (cancer, reproductive organ disorders, hormonal disorders, libido dysfunction, sexual orientation, and birth defects in the offspring) were too small to be provide sufficient power to rule out increased risks. It is possible that there was a bias in subjects who were able to be located compared to those not located 30 years later, and there may have been inaccurate recall for some measures collected at follow-up. The measures of infertility were weak, and the clinical relevance of the subjective measure of menstrual bleeding is questionable. A possible weakness of the study is that it may be perceived as being biased towards the null hypotheses because it was funded by companies that sell soy formula.

Utility (Adequacy) for CERHR Evaluation Process: This report is of limited utility for the evaluation process. Additional follow-up in this cohort of women for age of onset of menopause is recommended based on other studies that show a reduction in primordial follicle formation in mice exposed to genistein during neonatal life (discussed in **Section 3.3**).

Zung et al., 2008 ***(580)*****,** support not indicated although authors state no conflicts of interest, conducted a cross-sectional study to compare breast development in female infants fed soy formula for at least 3 months to infants fed breastmilk or cow's milk-based formula. Female infants ≤2 years of age (n=694) were enrolled from 10 pediatric clinics in Isreal between January 2003 and December 2004. None of the infants were referred to a clinic for an issue related to breast development. The infants underwent a physical exam that included measurement of breast buds. Following the exam, parents were asked if the infant was breastfed or formula-fed and the ages at initiation and termination of each type of diet. Infants that were continuously fed soy formula for at least 3 consecutive months were enrolled in the "soy" group (n=92, 13.3%) while infants fed breastmilk or a cow's milk-based formula were enrolled together in the "milk" group (n=602, 86.7%). Infants ranged in age from 3.1 to 24 months. Prevalence and proportion data were analyzed with a chi-square test or by Fisher exact test. Differences in length of exposure in the soy group were analyzed by Kruskal-Wallis analysis of variance and the Dunn method for pairwise comparisons.

There was no difference in breast bud development between diet groups when age of the infant was not considered or when infants were compared in the first year of life. However, group differences were observed in the "second" year infants. In the "milk" group, breast bud prevalence significantly decreased from the first year to the second year (23.2% to 10.3%, $P<0.001$). Breast bud prevalence did not change in the "soy" group during this time (19.1% verus 22%). The prevalence of breast buds in the "soy" group was significantly higher than in the "milk" group (OR=2.45, 95% CI=1.11–5.39). None of the infants had other signs of sexual development such as pubic hair.

The majority of infants in the "soy" group (75/92) also consumed cow's milk-based formula, breast milk, or both prior to visiting the clinic. The duration of soy formula feeding was shorter in these infants compared to those exclusively fed soy formula. However, there were no significant differences in breast bud prevalence between these soy formula subgroups. No differences in breast bud prevalence were observed when the length of soy formula exposure or starting age of soy formula consumption were considered. Breast bud prevalence in the breastfed and milk-based formula subgroups were also similar, 13.4% and 19.4%, respectively. The authors suggest that the differences in breast bud prevalence between the "soy" and "milk" group in the second year of life is due to estrogenic soy isoflavones and that high endogenous estrogens during the first year may account for similar breast bud prevalence at that age. Alternatively, they suggest that isoflavones may have partial antagonist effects on a background of high endogenous estrogens during the first year, but become agonistic in the second year when endogenous concentrations of estrogens are lower.

Strength/Weaknesses: This is a cross-sectional study; these have inherent limitations in their general design. Nonetheless, this appears to be a sound study conducted in a relevant and relatively homogenous population. The overall number of subjects is reasonable, although those enrolled in the "soy" group is relatively small and many of these subjects also consumed cow milk-based formula. Thus, an interaction between the different formulae cannot easily be discounted. The study was conducted over a relatively short time. Serum/urinary levels of soy isoflavones were not measured. Weaknesses outweigh the strengths in this study.

Utility (Adequacy) for CERHR Evaluation Process: This report is of no utility in the evaluation process.

3.2.1.6 Cholesterol

Cruz et al., 1994 ***(199)***, supported by NIH, the Perinatal Research Institute, USDA, and Ross Laboratories, conducted a prospective, partially randomized, controlled study to evaluate the effects of cholesterol and phytoestrogen intake on cholesterol-synthesis rates in infants. Thirty-nine normal term Caucasian male infants were recruited from area hospitals. Of the 33 infants who completed the study, 12 received human milk (cholesterol 2.59–3.88 mM) for the first 4 months of life, with occasional supplemental cow-milk formula (Similac® with Iron; cholesterol 0.28-0.85 mM). Twenty of the 26 remaining infants were randomized to receive either cow-milk formula or soy formula (Isomil® with Iron; cholesterol 0 mM). The remaining 6 infants were non-randomly assigned to receive a modified-soy formula with the same cholesterol concentration (0.28 mM) as the cow-milk formula. Infants received their assigned formulas beginning at age 3–7 days and continuing until age 4 months, with no additional nutrition received other than multivitamins. At 4 months, cholesterol-synthesis rates and urinary isoflavone excretion were determined, with measurements taken over a 4-day period. Serum cholesterol and lipid profiles were determined using validated enzymatic techniques. Serum LDL cholesterol concentrations were calculated from serum total cholesterol. Cholesterol fractional synthesis rate **[the percent cholesterol synthesized per day]** was determined from the rate of incorporation of deuterium into red blood cell-membrane cholesterol. Urinary isoflavones were determined by GC-MS. Data were analyzed using 1-way ANOVA with post hoc Tukey-Kramer test. Correlations between cholesterol intake and cholesterol fractional synthesis rates were determined using a nonparametric test for ordered alternatives.

Data for serum lipid profiles and fractional synthesis-rate determinations are shown in **Table 91**. The highest values for serum total and LDL cholesterol were found in breast-fed infants. These infants had the lowest fraction of synthesized cholesterol. There was a significant negative association between cholesterol intake and the fractional cholesterol-synthesis rate ($P<0.0001$). Isoflavone excretion was associated with soy-formula consumption. Multiple regression analysis controlling for group effect found total urinary isoflavone excretion to be negatively associated with fractional cholesterol-synthesis rate but not with serum cholesterol concentration.

Table 91. Serum Lipid Profiles and Fractional Cholesterol Synthesis Rates (Cruz et al., 1994)

Food	*Serum Concentration, mM*				*Fractional Cholesterol Synthesis Rate, %/day*
	Cholesterol			*Triglycerides*	
	Total	*HDL*	*LDL*		
Human milk	4.47 ± 0.20 [a]	1.32 ± 0.09	2.54 ± 0.2 [a]	1.30 ± 0.20	2.97 ± 0.49 [b]
Formula					
Cow milk	3.34 ± 0.24	1.22 ± 0.11	1.45 ± 0.25	1.44 ± 0.25	9.43 ± 1.12
Soy	3.11 ± 0.26	1.43 ± 0.12	0.99 ± 0.27	1.38 ± 0.27	12.02 ± 1.36
Modified soy	3.40 ± 0.28	1.26 ± 0.13	1.49 ± 0.29	1.39 ± 0.29	10.55 ± 1.05

Mean ± SEM.
HDL = high-density lipoprotein; LDL = low-density lipoprotein.
[a] Human milk value statistically different from all formula values.
[Authors presented different *P* values, all 0.03 or lower.]
[b] All values in column statistically different from one another, except cow-milk formula compared to modified-soy formula for which $P=0.1$.
From Cruz et al., 1994 *(199)*.

The study authors concluded that infants responded to different dietary cholesterol intakes through altered cholesterol-synthesis rates and that infant diet can affect serum lipid profiles, cholesterol-synthesis rates, and urinary isoflavone excretion.

Strengths/Weaknesses: After selection for breast feeding, random assignment to primary soy or cow-milk formula groups and documentation of no additional nutrition to 4 months are strengths. The inclusion of only males is a weakness. Selection bias was possible for breast feeding mothers. The modified soy-formula group was not included in randomization, and the multivariate analysis results were not described very clearly.

Utility (Adequacy) for CERHR Evaluation Process: This report is limited utility for the evaluation process.

3.2.1.7 Diabetes Mellitus

Fort et al., 1986 ***(581)***, funding not indicated, examined feeding histories of 95 diabetic children and controls to study the effect of breast feeding on the development of insulin-dependent diabetes mellitus. The feeding histories of the diabetic children were compared to those of a control group consisting of 194 non-diabetic siblings and an additional 95 non-diabetic friends living in the same geographic area. Data were analyzed by a sign test and chi-squared test. Eleven of the 95 diabetic children were excluded from statistical analysis because they changed addresses or could not be matched. No significant differences were detected in the incidence or duration of breast feeding among the groups, or in the introduction of solid foods. Almost twice as many diabetic children had been fed soy formulas compared to controls. **[Data on soy formulas were not shown. The authors noted that most children on formula received cow-milk formula and that the group differences in soy-formula intake were not statistically significant.]** The authors postulated that diabetic infants might have a higher incidence of gastrointestinal alterations giving rise to a switch to soy formula.

Strengths/Weaknesses: The case control design is a strength as is the use of sibling controls and unrelated controls matched by geographic area. Weaknesses include the retrospective collection of infant feeding information an average of 14 years later which could introduce problems with accurate recall, and the case control design could introduce recall bias, such that mothers of diabetic children might be more likely to recall soy formula use. The duration and quantity of soy-formula was not specified, e.g.,soy formula was said to have been combined with cow-milk formula in most instances. Specific numbers were not presented for soy formula; although more diabetic children were said have been fed soy formula than non-diabetic siblings or friend controls, however, this was said not to be statistically significant. Univariate analyses for group differences in soy formula intake were not shown and apparently no multivariate analyses were conducted.

Utility (Adequacy) for CERHR Evaluation Process: This report is of no utility for the evaluation process.

3.2.1.8 Cognitive Function

Malloy and Berendez, 1998 ***(582)***, funding not indicated, studied the relationship between breast feeding during infancy and cognitive performance in 9- and 10-year-old children. Cognitive testing and health results were obtained for children in a 1978–1979 birth cohort. All 518 infants in the study had received soy formula. Of these infants, 342 had been breast-fed and 176 had never been breast-fed. Dietary information was obtained through questionnaires completed by the parents. The

Wechsler Intelligence Scale for Children—Revised (WISC-R) verbal, performance, and full-scale intelligence quotients (IQ) were administered to children in their homes. Data were analyzed using chi-squared and Student *t* test. A linear regression model was used to adjust least-square means for potential confounding variables. Differences were considered significant at $P \leq 0.05$.

The researchers initially found higher WISC-R verbal and full-scale mean scores among the breast-fed children. After adjustment for maternal education, paternal education, and annual family income, the differences were no longer significant. The authors concluded that in this relatively socio-economically advantaged population, additional advantages of breast feeding might be more difficult to demonstrate.

Strengths/Weaknesses: The use of a birth cohort, standardized testing in school-age children within a narrow age range, and the large sample size are strengths. Weaknesses include the retrospective collection of infant feeding information only by questionnaire 9–10 years later and the comparison only between children who were breast-fed for any length of time and children who were exclusively soy formula-fed. If any real differences existed, they could have been diluted by soy exposure in the breast-fed group.

Utility (Adequacy) for CERHR Evaluation Process: This study has no utility for the evaluation process.

Jing et al., 2008 *(583)*, supported by the USDA, conducted a study to evaluate whether soy formula use affects normal brain development in young infants. Electroencephalographic (EEG) signals were collected from eight brain areas in each hemisphere from 3- and 6- month old male and female infants fed either milk-based formula (n=46) or soy formula (n=39). Participating infants were recruited from an ongoing longitudinal and prospective study on the long-term health consequences of early infant diet. Parents selected whether infants would receive a milk-based formula or soy formula **[specific formula not specified, but said to be co**mmercially available and containing docosahexaenoic acid]. Absolute spectral power, relative spectral power, and spectral edge frequency (SEF) were the spectral variables included in the analysis. Absolute spectral power provides an estimate of the amplitude of neural activity, relative power represents the distribution of spectral power across frequency bands, and SEF is the frequency point below which a specific percent of power is present, e.g., SEFs 85, 90, or 95% are often reported. Frequency domain contained 4 bands (0.1-3, 3-6, 6-9, and 9-12 Hz). Baseline EEGs were obtained over a number of sessions in the morning while infants were awake with eyes open in a dimly lighted, electrically shielded, and sound-attenuated testing room. Mean values were calculated from electrodes placed in 8 brain regions in both hemispheres: the prefrontal, frontal, central, parietal, occipital, anterior temporal, mid-temporal and posterior temporal areas. Artifact-free and non-overlapping EEG segments were included in the analysis and ~ 6 to 8 non-overlapping segments were obtained from each EEG record. Differences in spectral power were analyzed by mixed-type ANOVA with independent variables of formula group and sex and dependent variables. Post hoc tests were used to detect differences between specific means.

Significant differences in spectral variables were observed for sex, age, and brain region.Highest spectral power was observed in the low frequency band, < 6 Hz, and in the frontal and anterior temporal brain areas. Spectral power at 6 months was generally higher than at 3 months of age. SEF was generally higher in female infants than male infants, especially at 6 months of age. None of the spectral variables differed between infants fed soy formula and milk-based formula.

Strengths/Weaknesses: The study was conducted in human infants that were consuming either milk-based or soy formula for at least one month prior to the tests. The use of human infants is strength and so is the route of administration. However, one month may not be a long enough treatment time to note differences. Other weaknesses include the fact that the amount of soy consumed is not known, nor is the diet brand name given. The sample size is relatively small (n=85) for this type of study. Moreover according to Table 1 only 66 subjects participated and in particular only two infant boys on soy diet were in the study. No diet effects were found, which may be due to the sensitivity of this rather gross measure of neural activity. Yet, sex and age effects were noted. Given the number of statistical comparisons it is likely they over report the significant differences but this is hard to know because they did not report what kind of post hoc pair-test statistic they employed.

Utility (Adequacy) for CERHR Evaluation Process: This study has no utility for the evaluation.

3.3 Experimental Animal Studies on the Individual Isoflavones Found in Soy Formula

3.3.1 Growth, Reproductive System and Endocrine-Related Endpoints

Summaries of studies describing developmental effects in experimental animals exposed during pre- or postnatal development are subdivided according to the following general endpoints: "Growth, Reproductive System, and Endocrine-Related," "Mammary Gland Development and Carcinogenesis," "Brain and Behavior," and "Other (Thyroid, Immune, Bone, etc.)." Within each health effect category, studies in mice are presented before studies in rats. Within each species, studies are separated according to timing of exposure (prenatal only, postnatal only, pre- and postnatal). In each of these timing of exposure categories, studies of females only are presented before studies of males only and studies of both sexes. Studies with oral exposure are discussed before parenteral exposures for studies in the same categories.

3.3.1.1 Mice: Prenatal only

3.3.1.1.1 Prenatal - Female Mice (oral)

Chan et al., 2009 ***(584)***, **[support not indicated]**, examined the effects of genistein on mouse oocyte maturation, and subsequent pre-and postimplantation development, both *in vitro* and *in vivo*. ICR mice were acquired from the National Laboratory Animal Center (Taiwan) and maintained on Harlan Teklad breeder chow **[bedding not described] [Purity of the genistein not stated]**. *Genistein treatment of oocytes:* Cumulus-oocyte clusters (COCs) were obtained via two different regimens: 1) directly from the ovaries of 21-day old mice, or 2) from the oviducts of 21-day mice; COC maturation was analyzed after treatment with or without various concentrations of genistein (1, 5, or 10 μM) for 24 hours **[number of animals, oocytes or oocyte clusters not stated] [basis of dose selection not stated]**; the oocytes were then denuded, fixed and the nuclear structures were visualized using phase-contrast microscopy. *Maternal exposure to genistein in the drinking water:* 6-8 week old female ICR mice were randomly divided into 4 groups (n=20/group) and offered the standard diet and genistein (0, 1, 5, or 10 μM) in the drinking water for 4 days COCs were collected **[site of collection not stated]** and analyzed for oocyte maturation, *in vitro* fertilization and embryonic development: cell proliferation was analyzed by dual differential staining, a TUNEL assay of blastocysts was conducted, blastocyst development after embryo transfer was evaluated, and total RNA of the blastocysts was extracted and

real-time PCR was performed to quantify expression of the retinoic acid receptors (RAR) α, β and γ. Data were analyzed using one-way ANOVA and *t tests*.

Genistein treatment of oocytes during in vitro maturation: A dose-dependent lowering of the maturation rate was observed in the genistein-treated oocyte groups. The ability of oocytes to be fertilized was significantly lowered (dose-dependent) after treatment of the oocytes with genistein during *in vitro* maturation (IVM). Genistein IVM pretreatment caused an injury effect as evidenced by a lower rate of cleavage to the two-cell stage (dose-dependent); in addition, the number of embryos which cleaved to form blastocysts in the genistein-treated groups was significantly lower than that of the untreated controls (dose-dependent). The total number of blastocysts was less (dose-dependent) following genistein treatment during IVM of oocytes. The number of trophectoderm (TE) cells in the blastocysts was also lower during IVM upon genistein pretreatment of the oocytes (dose-dependent). Genistein application did not affect the number of ICM (inner cell mass) cells present in the blastocysts; however, the ICM-total cell ratio was higher in blastocysts derived from the groups treated with genistein during IVM when compared to the untreated controls. TUNEL staining revealed enhanced apoptosis of blastocysts of the genistein-pretreated oocyte groups (dose-dependent). The implantation ratio of blastocysts derived from oocytes treated with 10 μM genistein (high dose) was significantly lower than that observed from the control blastocysts. The proportion of implanted embryos that failed to develop normally was markedly higher in the 10 μM genistein-pretreated group vs. the control. The genistein-pretreated groups displayed a higher resorption rate than the untreated control group and the placental weights of blastocysts derived from genistein-treated oocytes in IVM were significantly lower than the control group. The average expression levels of RARα and RARγ in the blastocysts did not vary significantly across the three groups. However, RARβ expression in the blastocysts derived from genistein-treated oocytes was significantly lower than that in the control blastocysts. Maternal exposure to genistein in the drinking water induced significant injury to oocyte maturation and fertilization, resulting in inhibition of embryonic development from the zygote to blastocyst stage.

Authors' conclusion: Exposure of oocytes to genistein during *in vitro* maturation reduces the potential of postimplantation development. Consumption of drinking water containing genistein led to decreased oocyte maturation and *in vitro* fertilization, as well as early embryonic development injury. Moreover, the findings support a degree of selective inhibition of retinoic acid receptors in blastocysts treated with genistein during oocyte maturation.

Strengths/Weaknesses: Strengths of this study are the use of multiple dose levels of genistein at concentrations representative of human exposure, as well as an adequate number of animals in each treatment group. The outcome measures used are appropriate for the questions asked and provide mechanistic insight into the actions of genistein on oocyte maturation and embryo development. A weakness of the study design was maintenance of the animals on a phytoestrogen containing diet while attempting to assess the effect of genistein in the drinking water. Thus the actual genistein dose is probably underestimated. While the doses of 1, 5 and 10 μM in the diet or drinking water are suitable the rational for these concentrations in the culture media is unclear. The rational for a short dosing period is unclear. Finally, the relevance of these findings for human health is also uncertain.

Utility (Adequacy) for CERHR Evaluation Process: This study is of limited utility for the evaluation process based on use of multiple dose groups, robust sample size and assessment of outcome measures

that provide some useful mechanistic insight into the potential actions of genistein on oocyte maturation and embryo development. Use of a phytoestrogen diet and the difficulty is determining the relevance of these findings for human health limit the utility of this report.

3.3.1.1.2 Prenatal - Female Mice (non-oral)

Nikaido et al., 2004 ***(585)***, in a study supported by the Japanese Ministry of Health, Labor, and Welfare, examined the effects of prenatal genistein by sc injection on endocrine-sensitive tissues of female CD-1 mice. Mice were fed NIH-07 (a low phytoestrogen diet), and beginning on GD 15 (plug day not specified), were sc injected with 0 (DMSO), 0.5, or 10 mg/kg bw/day genistein (≥99% purity) for 4 days. **[The control group contained 6 dams/group, but it is not clear if that was the number of dams in treated groups. Although not explicitly stated in any of the tables, many of the results presented in the text indicate that there are 6 dams/group in the various treatment groups.]** Female offspring were weaned at 21 days of age. Onset of vaginal opening was monitored. Vaginal smears were assessed in 12 mice/group from 9 to 11 weeks of age. Six mice/group were killed and necropsied at 4, 8, 12, and 16 weeks of age, and histopathological evaluations were conducted on ovaries, uterus, vagina, and mammary glands. Whole-mount preparations of mammary glands were also examined. Data were analyzed by ANOVA, Kruskal-Wallis non-parametric test, and/or Fisher's Least Significant Difference test.

Prenatal genistein exposure accelerated body weight gain. At 16 weeks of age, body weight gain was **[~57%]** greater in the low-dose group and **[~66%]** greater in the high dose group compared to controls, as determined from a graph by CERHR. **[A full quantitative assessment of accelerated body weight gain via repeated measures ANOVA would have been more informative than the presentation provided in the manuscript.]** Vaginal opening was significantly accelerated by 1 day in the low-dose group and by 0.5 day in the high-dose group. Genistein exposure significantly increased estrous cycle length by 1.2 days in the low-dose group and 2 days in the high-dose group ($P<0.01$ for both dose groups). Changes in estrous cycle length resulted from prolongation of diestrus. The percentage of time (mean ± SEM) the mice spent in diestrus was 24.2 ± 2.1% in the control group, 31 ± 1.7% in the low-dose group, and 34.5 ± 1.8% in the high-dose group ($P<0.01$ for both dose groups). At 4 weeks of age, 6/6 control and low-dose mice had corpora lutea, while 2 of 6 high-dose mice had no corpora lutea. All control and genistein-treated mice had corpora lutea at later time periods. Mammary alveolar differentiation was more advanced in 2 of 3 mice with corpora lutea at 4 weeks of age. There were no differences in mammary development at later time periods. The study authors concluded that genistein exposure at doses equivalent to and 20-times higher than human exposure levels resulted in transient changes in the reproductive tract and mammary gland. Transient effects on the reproductive tract and mammary gland were also observed with bisphenol A and diethylstilbestrol, while prolonged effects were induced by zearalenone.

Strengths/Weaknesses: The use of very pure genistein, DES as a positive control, and low-phytoestrogen chow are strengths of this study. In addition, the dose levels used were relevant for human exposures. Weaknesses include the use of only 2 genistein dose levels, examination of only a small portion of prenatal development (GD 15–18), the lack of clarity on the number of animals treated per group, the lack of use of the litter as the experimental unit, and the small number of animals evaluated at each time point.

Utility (Adequacy) for CERHR Evaluation Process: This study is of limited utility for the evaluation because of the relevance of the dose levels of genistein used and the outcome measures are relevant

to reproductive function. Limitations for the evaluation of soy infant formula are use of a sc route of administration and treatment during gestation only.

Akbas et al., 2007 ***(336)***, supported by the National Institutes of Health, investigated the effects of genistein and daidzein on *Hoxa 10* expression in the uterus after *in utero* or adult exposure via ip injection in CD-1 mice. The mice (Charles River, Wilmington, MA) were fed Purina chow **[bedding not described, age not stated, purity not stated for any compound]**. Three experiments were conducted: *in utero* mouse exposure, adult mouse exposure, and reporter gene expression in Ishikawa cells. The results of the *in utero* exposure experiment are presented here and will be considered for in the evaluation. The adult exposure and *in vitro* experiments are presented as supporting information.

In utero exposure: Six groups of mated mice were treated via ip injection from the day after mating through parturition with DMSO (vehicle), 0.2 or 2 mg/kg bw/day of genistein, 2 mg/kg bw/d of daidzein, 17β-estradiol (0.5 mg/kg bw/d) or a combination of 17β-estradiol and genistein. Each group included 10 female offspring from at least 6 separate litters. The uteri of the two-week old female pups were collected and examined for histological anomalies; immunohistochemistry was performed for *Hoxa 10* expression, and real time RT-PCR was performed.

No gross or histological changes were seen and there was no effect on the *Hoxa 10* expression levels or the *Hoxa 10* spatial boundaries of expression compared to the control animals.

Adult exposure: On day 0, bilateral ovariectomy (OVX) was performed, on day 14, the mice were randomly assigned to six groups: control (DMSO), E2 (0.5 mg/kg), genistein (2 mg/kg), daidzein (2 mg/kg), genistein + E2, or daidzein + E2. The compounds were administered via a single intraperitoneal injection; all mice were killed 8 hours after injection. Uterine weights from the control, E2, genistein and daidzein groups (n=3) were normalized to the average of the control mice, results were analyzed via the Student's t test for assessment of uterotrophic changes. Hoxa 10 mRNA expression was measured for all groups (n=8) using real-time RT-PCR, normalized to actin expression, results were presented as fold induction and analyzed by ANOVA on ranks and post hoc Dunn's test.

Uterine weights were significantly higher the E2 and genistein groups compared to the control group; daidzein did not alter uterine weight compared to controls. E2 repressed Hoxa 10 mRNA expression and genistein induced Hoxa 10 mRNA expression; daidzein did not affect Hoxa 10 expression compared to controls. The combination of E2 and genistein resulted in Hoxa mRNA expression at a level intermediate between that induced by either agent alone. C3 expression was significantly greater in the E2 group; genistein exposure resulted in only a minimally greater C3 mRNA expression. Both ER-α and PR expression were lower for E2 and also for genistein by a similar amount. E2, but not genistein, induced proliferating cell nuclear antigen expression significantly more than the control. *In utero* exposure: No gross or histological changes were seen; there was no effect on the Hoxa 10 expression levels or the Hoxa 10 spatial boundaries of expression compared to the control animals.

Reporter gene expression: Ishikawa cells were transfected with the pGL3 promoter/ Hoxa 10 ERE construct. Genistein and daidzein were used to treat cells at various concentrations **[concentrations not specified],** after 12 hours, luciferase activity was measured in the cellular lysate. Electrophoretic mobility shift assay was used to assess ER-α and ER-β binding to the Hoxa 10 ERE in the absence and presence of genistein and daidzein.

Ishikawa cells treated with genistein demonstrated a higher luciferase activity (dose-responsive) when compared to controls; those treated with daidzein demonstrated higher luciferase activity only at concentrations above the biologically relevant range. The addition of 10-6 M daidzein or genistein did not alter ER binding to the Hoxa 10 ERE.

Authors' conclusion: In utero exposure to isoflavones is unlikely to result in *Hoxa 10*-mediated developmental anomalies. Adult genistein exposure alters uterine Hoxa 10 expression, a potential mechanism by which this agent affects fertility.

Strengths/Weaknesses: Strengths of the study include the selection of reasonable dose levels and appropriate time point of assessment. Weaknesses of the study are that the dose levels and groups were not adequately described. Also, the strategy used to select offspring and control for litter effects was not clearly described. The authors could have evaluated many more developmental, estrogen-related genes using the PCR approach. Assessment of uterine wet weight and PCNA staining would have been useful endpoints to include in the study. The weakness of the ER and PR measurements were problematic because the positive control (E2) did not produce the expected outcome. The biological relevance of the outcome is not known because no reproductive function end point was measured.

Utility (Adequacy) for CERHR Evaluation Process: This study is of limited utility in the evaluation. The study could have higher utility if additional outcome measures were included, i.e., additional estrogen-related genes, uterine weight wet and PCNA staining.

Chan et al., 2007 ***(586)***, [source of funding not stated], used an *in vitro* system of cultured mouse blastocysts to investigate the cytotoxic effects of genistein (25 or 50 µmol/L for 24 hours). Apoptosis was determined using the TUNEL assay and Annexin-V and propidium iodide staining were used to determine apoptotic or necrotic cells. Treatment of blastocysts with genistein resulted in a dose dependent increase in apoptosis, fewer cells primarily in the inner cell mass, no effect on "implantation", and decreased progression to later developmental stages. Similar methods were used in a single study to assess blastocysts recovered from females exposed to 10 µmol/L genistein in drinking water for 4 days following mating. Results of this study showed increased apoptosis and decreased cell numbers in blastocysts. The authors conclude that their results provide evidence that genistein could have teratogenic effects through the induction of apoptosis.

Strengths/Weaknesses: The strength of this study was use of two apoptosis-related endpoints. A major weakness of the study is inadequate description of experimental details, including rationale for dose selection, staging of blastocyst development, and assessment of implantation. In addition, the actual dose consumed was not controlled for and the authors overstate the conclusions.

Utility (Adequacy) for CERHR Evaluation Process: This study is of no utility for the evaluation process due to inadequate description of experimental details and a lack of discussion on the relevance of dose selection.

3.3.1.1.3 Prenatal - Male Mice (oral)

Vilela et al., 2007 ***(587)*****, [support not indicated]**, assessed the effects of oral gavage of genistein, vinclozolin, or a mixture of the two effects on hypospadias in male mice. Timed-pregnant female CD1

mice (CRL, Wilmington, MA) were received on gestation day 8 (GD 8), housed with laboratory grade pine shavings, and fed a soy-free diet chow. The mice were assigned to 4 groups (n≥8 mice/group) **[basis of assignment not stated]**: Group 1 received the vehicle (corn oil), Group 2 received 0.17 mg/kg bw/day of genistein **[purity not stated]**, Group 3 received 10 mg/kg bw/day of the fungicide vinclozolin **[purity not stated]**, and group 4 received both genistein and vinclozolin at the same levels from GD 13 through GD 17. The dose level for genistein was chosen on the basis of the realistic daily exposure levels of genistein, as determined by the authors according to the amounts of genistein in various foods; the dose level for vinclozolin was based on the US EPA's report of the lowest observed effect level of 11.5 mg/kg bw/day for acute exposures. On GD 19, the fetuses were harvested and the sex was determined; the determination of hypospadias was made by expressing the bladder contents and noting where they emerged from the urethral opening. The genital tubercle from ~ ½ of the fetuses was saved in NBF, embedded in paraffin and slides were stained with H&E. Hypospadias frequencies among the groups were compared using Fisher's exact analysis of two-by-two contingency tables.

The frequency of hypospadias overall was 0% in the control group (n=30), 25% in the genistein groups (n=24), 42% in the vinclozolin group (n=26), and 41% in the combination group (n=29).

Authors' conclusion: Genistein alone, vinclozolin alone, and the combination of the two resulted in a significantly higher frequency of hypospadias compared to the control group. Thus, simultaneous maternal consumption of genistein and vinclozolin, such as can occur in a nonorganic vegetarian diet, might result in an increase in hypospadias frequency.

Strengths/Weaknesses: Strengths of the study are administration of genistein by oral gavage and the use of a dose relevant to human consumption. Weaknesses are the assessment of only a single dose level of genistein, the absence of control for litter in calculation of the incidence of hypospadias, and the lack of additivity in the vinclozolin and genistein group. The data do not support the conclusion that simultaneous consumption of genistein and vinclozolin increases the frequency of hypospadias.

Utility (Adequacy) for CERHR Evaluation Process: This study is of limited utility for the evaluation due to the weaknesses in the study design.

3.3.1.1.4 Postnatal - Female Mice (oral)

Carter et al., 1955 ***(588)***, supported by the Tennessee Valley Authority, fed weanling-aged female Swiss mice (n=36/group) a diet containing commercial soybean meal, methanol-extracted soybean meal (controls), or methanol-extracted soybean meal to which genistin **[purity not given]** was added at 2 g/kg feed **[2000 ppm]**. The mice were weaned to these diets at weights of 9.4–12.1 g, which was estimated to be at 3 weeks of age. The diets were continued for 4 weeks. Females, housed 3/cage, were observed for vaginal opening. One male was placed in each cage with 3 females for 21 days during which time treated feed was continued. **[Males had been raised on Purina Laboratory Chow. Assuming a mature female mouse eats 0.18 kg feed/kg bw/day *(313)*, genistin intake would have been 360 mg/kg bw/day.]** Statistical methods were not discussed. No alterations in feed consumption or body weight gain per cage were detected. Vaginal opening was advanced in the genistin-treated group. Fifty-nine percent of genistin treated females produced litters compared to 82% of control females **[*P*=0.06, Fisher's exact test by CERHR]**. No effect of treatment on litter size and weight was detected. The authors concluded that genistin had adverse effects on female reproduction in mice, although they could not exclude an effect on the male during the cohabitation period.

Strengths/Weaknesses: Strengths include administration of genistein by the oral route and adequate numbers of animals. The study also appeared to control for litter effects. Although, administration of genistin in the diet is a strength; the group housing prevented determination of actual consumption. The lack of information on the age of the females at weaning, the use of a single dose level, failure to evaluate the stability or homogeneity of the dose in feed, exposure of the male during the cohabitation period, and the lack of evaluation of ovarian and uterine histopathology are weaknesses. Also, the publication did not report the types of statistical analyses that were performed and lacked proper control groups.

Utility (Adequacy) for CERHR Evaluation Process: This study is of no utility in the evaluation process because it is lacking in proper controls in the experimental design.

East, 1955 ***(589)***, from the Australian National Institute for Medical Research, conducted a series of three studies to examined reproductive endpoints in female mice consuming synthetic genistein **[purity not specified]** beginning near the time of weaning. Only one of the three studies included developmental exposure and is considered appropriate for inclusion in the Expert Panel's evaluation of soy formula. The other 2 experiments described in the publication focus on reproductive function assessment in mice exposed as adults and are only presented as supporting information.

In the first study, 15 weanling "Fawn Farm" strain mice (18 days old) per group were fed 0 or 2 mg/day genistein through stock diet for 21 days. **[Based on EPA *(313)* assumptions for female weanling B6C3F$_1$ mouse body weight (0.0110 kg), genistein intake would have been ~180 mg/kg bw/day. The composition of stock diet was not specified. In addition, the author noted that it was difficult to quantitate feed intake.]** Following the exposure period, the mice were fed stock diet for 14 days. Inspections for vaginal opening were conducted daily. Vaginal smears were conducted daily following vaginal opening. Data were evaluated by modified *t* test. Genistein significantly advanced vaginal opening compared to the control diet; mean ± SD number of days for vaginal opening post weaning were 5.47 ± 1.13 in the genistein group and 10.80 ± 3.61 in the control group. Cornified cells were seen immediately, and leukocyte infiltration was observed sporadically in smears from the genistein group. Mice cycled normally 5 days after transfer to stock meal **[data not shown]**. There was no detectable effect of genistein intake on body weight.

In the second study, 1-month-old female mice were castrated. At 2 months of age, 10 mice per group were treated with 0, 5, or 10 mg/day genistein through diet for 14 days and vaginal smears were conducted daily. **[Based on EPA assumptions *(313)* for female B6C3F$_1$ mouse body weight in subchronic studies (0.0246 kg), genistein intake was estimated at 200 and 400 mg/kg bw/day in the high- and low-dose group, respectively.]** Leukocyte infiltration was comparable in smears from genistein-treated and control mice. However, cornified cells were seen in smears from 5 mice in the 10 mg/day group 1 week after treatment. Cornification persisted for 2–5 days. **[Although the study authors concluded that doses producing vaginal cell cornification were equivalent in immature and castrated animals on a body weight basis, the CERHR genistein estimate for castrated animals in the 10 mg/day group was twice that for immature animals.]**

In the third study, fertility was evaluated in 2-month-old male and female mice. Males included in the study were demonstrated to be fertile and females had regular estrous cycles for 14 days. Ten male and female mice per sex were given genistein 15 mg/day through diet, and 20 male and female mice per sex

were fed control diets for 10 days prior to mating. **[Based on EPA assumptions for male and female B6C3F$_1$ mouse body weight in subchronic studies (0.0316 and 0.0246 kg, respectively), genistein intake was estimated at 470 and 610 mg/kg bw/day in males and females.]** Treated animals were paired 1 to 1 with untreated animals, and controls were paired together. Treated females were mated twice and treated males were mated once during the time period for which they continued to receive genistein. Genistein treatment lasted 31–55 days in females and 22–25 days in males. At the end of the treatment period, males and females were returned to stock diet and mated twice more with respective partners. Control animals were mated a total of three times. Litters born during the treatment period were discarded, while litters born after return to stock diet were left undisturbed until weaning. Parameters evaluated included fertility, matings, number of litters born, litter size, and pup mortality. **[It does not appear that statistical analyses were conducted.]** Sterility was defined as lack of mating, and infertile matings were defined at those resulting in pseudopregnancy, resorptions, or abortions. Treatment of female mice with genistein resulted in cornification of vaginal smears within 3 days, and mice remained in estrus during the remaining 7 days prior to mating. Results for breeding parameters are summarized in **Table 92**. The most prominent effect observed in treated female mice was an increased number of stillborn pups. The effect resolved after the treatment period ended. Genistein treatment adversely affected fertility in males as noted by increased sterility and infertility. There was some recovery, albeit incomplete, in male fertility after genistein treatment ended.

Table 92. Breeding Performance in Male and Female Mice Exposed to Genistein (East, 1955)

Parameter	*Treated Female × Untreated Male*		*Treated Male × Untreated Female*		*Untreated Male × Untreated Female*
	During Treatment	*After Treatment*	*During Treatment*	*After Treatment*	
Sterile pairs, n	2	0	5	2	0
Matings, n	16	20	5	16	30
Infertile matings, %	25	35	60	38	17
Litters born, n	12	13	2	10	25
Litters weaned, n	N/A	13	N/A	10	21
Pups born, n	56	77	15	70	192
Pups stillborn, n	23	0	0	0	0
Litter size at birtha	4.7	5.9	7.5	7.0	7.7
Litter size at weaning[a]	N/A	5.4	N/A	5.6	7.0
Weaning weight, g[a]	N/A	8.2	N/A	6.8	7.3
Pups weaned, %	N/A	91	N/A	80	77

n = 10 pairs per mating condition.
Females were mated twice during the treatment period and twice after returning to control diet.
Males were mated once during the treatment period and twice after returning to control diet.
Controls were mated three times.
[a] Mean.
N/A = Not applicable
From East, 1955 *(589)*.

Strengths/Weaknesses: A strength of this historical study is that adequate numbers of animals were used. Additionally, genistein was administered via the oral route. A weakness is that exposure was

unclear due to administration of genistein through diet. Because no information was provided on daily intake by mice, exposure doses could only be estimated. The very high genistein dose level (~200 mg/kg bw/day) did not mirror general human exposure levels. No statistical analysis was performed. Endpoints examined were limited to vaginal opening and smears and fertility.

Utility (Adequacy) for CERHR Evaluation Process: This developmental exposure portion of this study is of no utility in the evaluation process because of the high genistein dose levels used and the few endpoints examined.

Jefferson et al., 2009 ***(217)***, supported by the Intramural Research Program of NIEHS/NIH and Oak Ridge Institute for Science Education, compared the estrogenic effects of oral exposure to genistein and its conjugated or glycoside form, genistin, in neonatal mice. Adult female CD-1 mice were obtained from Charles River Breeding Laboratories (Raleigh, NC), fed NIH 31 mouse chow and housed on hardwood chip bedding **[not further described]**. The females were bred to male mice of the same strain and allowed to deliver their litters. On PNDs 1–5 (total 5 doses) female pups received one of the following three treatments:

1. Daily sc injection of 0, 12.6, 20 or 25 mg genistein/kg/day.
2. Daily oral administration of 0, 25, 37.5, or 75 mg genistein/kg/day.
3. Daily oral administration of 0, 10, 20, 40, or 60 mg genistin/kg/day (equal to 6.25, 12.5, 25, or 37.5 mg/kg/day in genistein aglycone equivalents)

All test substances were suspended in corn oil which also served as the control; oral dosing was accomplished by inserting a pipet inside the mouth of each pup. Genistein and genistin were both 98% pure **[basis of dose selection not described]**. Genistin is composed of genistein with a large sugar group (37.5% of its molecular weight); for reporting purposes, the genistin dose levels were adjusted to genistein (aglycone) equivalents to be 0, 6.25, 12.5, 25, and 37.5 mg genistein/kg/day.

Uterotropic Bioassay: Four hours after the last treatment (PND 5), pups were killed and the uterus was collected, weighed and frozen (n=8 pups/group). RNA was isolated (minimum 4 per group) and reverse transcribed into cDNA. Lactoferrin (LF) expression was determined by real-time RT-PCR as verification of estrogenic activity. *Serum Levels of Genistein:* Trunk serum was collected from pups in the high dose oral genistein and oral genistin groups at each of the following time points (n=4–6 pups/time point): 0, 0.5, 1, 2, 4, 8, 24 and 48 hours after the last treatment on PND 5. Total genistein content, and daidzein and equol levels were determined by LC-ES/MS/MS. *Ovarian Histology:* Ovaries were collected from PND 19 prepubertal mice treated orally with genistin (n=8 mice/group); this time point is after secondary follicle formation, but before corpora lutea formation. Ovaries were processed for histology and 3 levels of each ovary were scored of the presence and number of multioocyte follicles (MOFs) and evaluated for alterations in ovarian morphology. *Vaginal Opening, Estrous Cyclicity, and Fertility*: Mice treated orally with genistin were evaluated daily for vaginal opening beginning on PND 22 (n=16 mice/group). At 2 months of age, daily vaginal smears were obtained from half of these mice (n=8 mice/group) and examined to determine the stage of the estrous cycle. At 2, 4 and 6 months of age, the females (n=16 mice/group) were paired with proven control males of the same strain for a two-week period and assessed for fertility parameters. *Statistical Analysis*: Uterine weights and real time RT-PCR data were analyzed by ANOVA followed by Dunnett's test. MOF, estrous cyclicity and

fertility end points were analyzed using the nonparametric Mann-Whitney tests or Fisher's Exact test. The Cochran-Armitage trend test was used to test for dose trends in quantal responses.

Uterotropic Bioassay: PND 5 pups treated with 20 and 25 mg/kg bw/day sc injection of genistein had greater uterine weights than their controls; greater uterine weights were also observed in the pups treated with 25 and 37.5 mg/kg bw/day oral genistin. Compared to the sc injection dose of genistein, approximately 20-33% more of the oral genistin was needed to elicit similar uterine weight changes. No differences in uterine weights were seen following oral genistein at 25 or 37.5 mg/kg bw/day; uterine weights were only slightly higher in the 75 mg/kg bw/day oral genistein group than its control. *Serum Levels of Genistein:* Levels of daidzein and equol were found to be consistently undetectable. The total and aglycone levels of genistein in serum following oral treatment with 37.5 mg/kg genistin suggest that the glucoside moiety of genistin is readily cleaved to the aglycone form, genistein, which can either be absorbed into the circulation as the aglycone (C_{max}=5.6 μM) or conjugated in the gut and secreted as conjugated forms into circulation (total C_{max}=19.2 μM). The dose-adjusted AUC for total genistein was slightly lower following oral genistin (83% of sc injection of genistein), and the AUC for the aglycone adjusted for dose (43% of sc injection of genistein). The internal exposures following orally administered genistein were much lower compared to oral genistin, measured either as C_{max} or AUC. *Ovarian Histology:* At PND 19, the percentage of multioocyte follicles (MOFs) rose with the dose of oral genistin and was significantly higher than controls in all groups except the lowest dose. *Vaginal Opening, Estrous Cyclicity, and Fertility:* Mice treated with the highest oral dose of genistin (37.5 mg/kg bw/day) had delayed vaginal opening–50% of these mice achieved vaginal opening two days later than their age-matched controls. In addition, a few mice in the top two dose groups did not have definitive vaginal opening even 5 days after the last control mouse exhibited opening. Day of vaginal opening was comparable to the controls for mice in all other groups. None of the mice in the control group had abnormal estrous cycles whereas 38% of 12.5 mg/kg bw/day group, 62% of the 25 mg/kg bw/day group, and 88% of the 37.5 mg/kg bw/day group had abnormal cycles; this difference was significant for the 25 and 37.5 mg/kg bw/day groups. The abnormal estrous cycles were predominately due to prolonged estrus. The percentage of oral genistin-treated females that mated (vaginal plug positive) was similar between dose groups at 2, 4 and 6 months of age. There was a significant reduction in the number of mice delivering live pups in the 25 and 37.5 mg/kg bw/day treatment groups. Mice in the high-dose group that did deliver had fewer pups at 2 and 6 months of age and mice in the 25 mg/kg bw/day group had fewer live pups at 6 months of age. A summary of fertility endpoints combined across the time points is presented in **Table 93**:

Table 93. Fertility Endpoints Following Oral Exposure to Genistin (Jefferson et al., 2009)

Endpoints	*Dose of Oral Genistin (mg/kg bw/day)*				
	0	*6.25*	*12.5*	*25.0*	*37.5*
Number of litters with live pups per dam	1.8	1.9	1.4	1.6	1.3*
% Plug positive females delivering live pups	100	89.3	84.4*	66.7*	63.9*
Total number live pups per dam (over all three time points)	23.5	26.5	17.9	21.2	13.8*
Average number of live pups per litter	13.3	13.2	12.0	11.8	9.3*

* Significantly different than the control group.
From Jefferson et al., 2009 *(217)*.

All control mice delivered their pups by early morning of gestation day 19 (GD 19). All oral genistin groups had some dams that delivered late in the afternoon of GD 19, or as late as GD 20 or GD 21. On-time deliveries were significantly reduced in the 25 and 37.5 mg/kg bw/day groups at all ages, and in the 12.5 mg/kg bw/day group at 4 and 6 months of age. At 4 and 6 months of age, over half the pups in the 12.5, 25 and 37.5 mg/kg bw/day groups had either late deliveries or no live pups. This effect was also seen in the 6.25 mg/kg bw/day group at 6 months of age with 27% of the dams exhibiting late delivery. Average pup weight was greater for the litters born 1 to 2 days late when compared to the pups born on time.

Authors' conclusion: The data support the idea that the dose of the physiologically-active compound reaching the target tissue, rather than the administered dose or route, is most important in modeling chemical exposures. This is particularly true with young animals where phase II metabolism capacity is under-developed relative to adults.

Strengths/Weaknesses: Strengths of the study include that two forms of the chemical were used (genistein and genistin); two modes of exposure were used (sc injection and oral); dose responses were given; a time course for endpoint evaluation was done; multiple relevant end points were measured; adequate numbers of animals were used. The only minor weakness was that exposure mimicked only neonatal exposure.

Utility (Adequacy) for CERHR Evaluation Process: The experiments where genistin or genistein treatment was by oral administration are of high utility as there were no major weaknesses. The experiment where genistein treatment was by sc injection is of limited utility due to the route of administration

3.3.1.1.5 Postnatal - Female Mice (non-oral)

Begum et al., 2006 ***(590)***, supported by the Japan Society for the Promotion of Science, investigated whether neonatal estrogenic treatments augment the incidence of complex atypical hyperplasias (CAH) and carcinomas in murine *PTEN* heterozygous (+/-) mutant mice. Male m*PTEN* +/- mice (129 Ola x C56BL/6) with a deletion of exons 3-5 of m*PTEN* were mated with wild-type C56BL/6 females (Seac Yoshimoto, Fukuoka, Japan) **[feed and bedding not described]**. Diethylstilbestrol (DES), genistein and estriol (E_3) were dissolved in ethanol and corn oil **[purity not stated for any compound]**. Female pups were treated with daily subcutaneous injections of DES (1 ng/g/day), genistein (50 µg/g/day), E_3 (4 µg/g/day) or vehicle alone from the 1st to the 5th day after birth **[basis of dose selection not stated]**. Sixteen m*PTEN* +/- pups and 16 wild-type pups were assigned to each treatment group. Eight m*PTEN* +/- pups and 8 wild-type pups in each treatment groups were killed on the sixth day (day after last dose), the remaining pups were weaned at 21-22 days of age and surviving mice were killed when they were 52 weeks old. The uterus was weighed and compared to the terminal body weight for both age groups. For the adults, one uterine horn was sectioned, stained with H&E for Histopathological evaluation, and the number of endometrial stromal cells was quantified. Total RNA was extracted from the other uterine horn and cDNA was generated. Quantification of PCR products was conducted using a fluorescence-based real-time detection method. Assay-on-Demand primers and probes were used for *Hoxa 10* and *Hoxa 11*. Means and standard errors were calculated and analyzed via protected Least Significant Difference of Fischer's test (ANOVA analysis).

Neonatal treatment with DES or E_3 significantly affected the ratio of uterine weight to terminal body weight (see **Table 94**). At 52 weeks of age, myometrial tumors were observed in one wild-type animal from the DES group, two m*PTEN* +/- animals from the genistein group and two m*PTEN* +/- animals from the E_3 group. Macroscopically, atrophic uteri were observed in both the E_3 and the DES treated mice.

Table 94. Mean Relative Uterine Weights following Neonatal Treatment with Genistein by SC Injection (Begum et al., 2006)

Treatment, age	% of Control Value		
	DES	Genistein	E_3
m*PTEN* +/– pups (6 days)	166*	134	188*
Wild-type pups (6 days)	163*	142	177*
m*PTEN* +/– adults (52 weeks)	45*	86	38*
Wild-type adults (52 weeks)	75	93	57*

*Statistically significant.
From Begum et al., 2006 *(590)*.

At histopathological examination, seven of the eight m*PTEN* +/- mice had multifocal CAH; age-matched wild-type mice did not develop these lesions. The incidence of CAH in m*PTEN* +/- mice was reduced by neonatal estrogenic treatment (88% of mice for control, 50% for DES, 38% for genistein, and 25% for E_3). The average number of focal CAH per uterine horn was significantly lower for all three treatments compared to the control. The incidence of adenocarcinomas in the m*PTEN* +/- mice treated neonatally with DES and E3 was significantly lower than the control group. There was no effect in the wild-type mice. Hyaline was deposited in the endometrial stroma in the m*PTEN* +/- mice with DES treatment (2/6 mice) and genistein treatment (4/8 mice) and in all wild-type mice. The endometrial stroma was atrophic in DES- and E_3-treated wild-type (1/8 and 2/8 mice, respectively) and m*PTEN* +/- mice (1/6 and 4/8). Vehicle-treated mice were unaffected. The density of the stromal cells was significantly lower in both m*PTEN* +/- and wild-type mice with DES, genistein and E_3 treatment when compared to the control. In m*PTEN* +/- mice treated neonatally with estrogenic agents, CAH were rare in the hyalized or atrophic area of the stroma, whereas in the same mice CAH developed in the areas containing sufficient stromal cells. *Hoxa* genes were highly expressed in the uteri of the wild-type mice and the m*PTEN* +/- mice from the vehicle group, but expression was significantly less for m*PTEN* +/-mice treated neonatally with estrogenic agents.

Authors' conclusion: Neonatal estrogenic exposure induced stromal atrophy and/or hyalinization accompanied by regressed expression of *Hoxa 10* and *Hoxa 11*, and exerted an inhibitory effect on *PTEN*-related tumorigenesis.

Strengths/Weaknesses: Strengths include the use of an established animal model, relevant timing (neonatal) of exposure, and the use of two different estrogenic compounds, DES and estriol, as positive controls. However, the relevance of single gene, non-conditional knockout models may be limited to inherited single gene loss/mutation in humans, which may not be common. Not clear if mutation in mouse model affects genistein PK/PD, or other endocrine factors that may interact with genistein. Not clear if an appropriate semi-purified diet was used and cannot exclude the possibility of highly variable other isoflavone exposures (which is likely if a standard lab chow was used). Number of events in each group is small for several endpoints, suggesting an underpowered study for some measures. Not clear relevance or basis of exposures. Any utility is probably limited to suggesting mechanism linked to PTEN loss, e.g., some endometrial cancers. Use of only one concentration of genistein prevents determination of dose-response relationships and is considered a weakness of this study. In addition the route of administration of test agents was not relevant to human health.

Utility (Adequacy) for CERHR Evaluation Process: The route of administration and use of only a single concentration of genistein limit the usefulness of the results of this study for the for CERHR evaluative process. The relevance of these results to human health is unclear. However, these data may be useful in informing other studies and shaping future investigations. Overall, the study has no utility in the evaluation.

Jefferson et al., 2002 ***(591)***, from NIEHS, examined the effects of neonatal sc injection treatment with genistein exposure on the mouse ovary. Female mice from different litters were pooled and redistributed to produce litters of 8 females. On PND 1–5 (day of birth=PND 1) 16 pups/group were sc treated with genistein in corn oil at 0, 1, 10, or 100 µg/day. Study authors estimated the doses at 0, 0.5, 5, or 50 mg/kg bw/day. The genistein treatment protocol was conducted in CD-1 mice, wild type C57BL/6 mice, and in ERα or ERβ knockout mice. Another group of CD-1 mice was exposed to the tyrosine kinase inhibitor lavendustin A at 1 or 10 µg/day on PND 1–5. CD-1 mice were killed on PND 5, 12, or 19, and knockout mice were killed on PND 19. Ovaries were removed and pooled together by treatment group. Ovaries were pooled from 8 mice on PND 5 and 12 and from 4 mice on PND 19. RNA and protein were extracted from some ovaries for measurement of *ER* expression by ribonuclease protection assay and Western blot. Additional ovaries were prepared for histologic examination in 8 mice/group and immunohistochemical staining for ERα and ERβ on PND 19. In another part of this study, mice were weaned on PND 21 and were treated on PND 22 with human chorionic gonadotropin hormone to induce superovulation. The numbers of ovulated oocytes within the oviduct were counted. **[With the exception of ovulation data, analyzed by Dunnett's test, statistical significance was not reported for any endpoint.]**

In ovaries from the control CD-1 mice, *ERβ* RNA was expressed at more than twice the level of *ERα* RNA and expression increased with age. Expression of *ERα* decreased with age. A 3-fold increase in *ERα* RNA expression was observed on PND 5 in the genistein 1 µg/day group, and a >2-fold increase in *ERα* RNA expression was noted on PND 12 in the 10 µg/day group. None of the genistein doses increased expression of *ERβ* by more than 1.5-fold. Treatment with genistein 100 µg /day reduced expression of *ERα* and *ERβ* RNA on PND 5, but the effect became less apparent on PND 12 and 19. **[Normalized data were not shown for the 100 µg/day group.]** The authors stated that Western blot and immunohistochemical analyses conducted on PND 19 confirmed the increased ovarian expression of *ERα*. However, in contrast to RNA expression, which peaked on PND 5 following genistein exposure, ERα immunoreactivity peaked on PND 19.

Immunohistochemical analysis revealed that ERα was localized in interstitial and thecal cells in control mice. Genistein treatment induced ERα in granulosa cells, with strongest induction occurring in the 1 and 10 µg/day groups. ERβ was strongly expressed in granulosa cells of controls. Genistein treatment resulted in no obvious changes in the location of ERβ expression.

C57BL/6 and *ERβ* knockout mice displayed the same patterns of ER expression as CD-1 mice with localization of ERα in interstitial and theca cells and ERβ in granulosa cells at PND 19 **[data not shown]**. Induction of ERα in granulosa cells occurred following treatment with genistein 10 µg/day in C57BL/6 and *ERβ* knockout mice but not in *ERα* knockout mice. Treatment of CD-1 mice with lavendustin A 10 µg/day, which has no known estrogenic activity, increased ERα immunoreactivity in granulosa cells on PND 19, although the effect was less than the effect produced by genistein. No

effect of lavendustin A treatment **[a highly specific inhibitor of tyrosine-specific protein kinases]** on ERβ immunoreactivity in ovary was detected **[data not shown]**. The study authors suggested that induction of ERα in granulosa cells is independent of a functional ERβ and may be partially induced by genistein inhibition of tyrosine kinase.

Ovaries from each strain of mice were evaluated for multi-oocyte follicles. As shown in **Table 95**, genistein treatment resulted in a dose-related increase in multi-oocyte follicles in CD-1, C57BL/6, and *ERα* knockout mice but not in *ERβ* knockout mice. Ovaries of mice in the 10 µg/day group had an increased incidence of atretic intermediate and large follicles (4.5 ± 0.4/ovary section in control group, 5.6 ± 0.3 in the genistein 1 µg/day group, and 9.1 ± 1.0 in the genistein 10 µg/day group **[variances not specified]**). No multi-oocyte follicles were observed in 8 CD-1 mice/group treated with 1 or 10 µg/day lavendustin A. The study authors concluded that genistein induction of multi-oocyte follicles appears to occur through an ERβ-related mechanism and not through inhibition of tyrosine-specific kinases.

Table 95. Multi-oocyte Follicles in Mice Treated with Genistein as Neonates (Jefferson et al., 2002)

Genotype	*Genistein Dose, µg/day*					
	0	*1*	*10*	*100*	*BMD_{10}[b]*	*$BMDL_{10}$*
CD-1	0/8 (0)[a]	1/8 (2)	2/8 (4)	6/8 (8)	20	12
C57BL/6	1/11 (1)	1/11 (1)	9/11 (3)	11/11 (10)	2	1
ERα knockout	1/3 (1)	2/4 (1)	4/6 (4)	N.D.	2	1
ERβ knockout	1/2 (1)	0/4 (0)	0/5 (0)	1/3 (2)	51	19

[a] Data expressed as number of mice expressing at least 1 multi-oocyte follicle in any section, (largest number of multi-oocyte follicles observed in a single section).

[b] The BMD_{10} is the benchmark dose associated with a 10% effect, estimated from a curve fit to the experimental data. The $BMDL_{10}$ represents the dose associated with the lower 95% confidence interval around this estimate. A 10% alteration in a continuously distributed parameter is an arbitrary benchmark that may not be comparable to a similar alteration in any other endpoint. The $BMD_{1\ SD}$, which represents an alteration equivalent to 1 SD of the control distribution, may permit more appropriate comparisons of the responses of continuously distributed parameters. Benchmark doses are used commonly in a regulatory setting; however, they are used in this report when the underlying data permit their calculation, and are only supplied to provide one kind of description of the dose-response relationship in the underlying study. Calculation of a benchmark dose in this report does not mean that regulation based on the underlying data is recommended, or even that the underlying data are suitable for regulatory decision-making. Values were calculated using the power model by CERHR using EPA Benchmark Dose Software version 1.3.2. The program offers models based on homogeneity of variance, and CERHR was guided by the program in this regard. A probit model was used for dichotomous variables.

N.D. = Not determined.

From Jefferson et al., 2002 *(591)*.

In the test to determine ovulation in 22–23-day-old mice, treatment with genistein 1 µg/day significantly increased numbers of ovulated oocytes (33.9 ± 3.3 compared to 23.2 ± 2.8 oocytes in control. **[The indicated variance is SEM (R. Newbold, personal communication August 17, 2005).]** There were smaller numbers of oocytes in oviducts of mice treated with genistein 10 and 100 µg/day (17.9 ± 1.4 and 16.5 ± 1.8 oocytes), but the results did not attain statistical significance. The study authors noted that the dose inducing increased ovulation coincided with the dose inducing increased *ERα* expression.

In summary, the study authors concluded that neonatal genistein exposure resulted in morphologic and functional changes in the mouse ovary. They concluded that the mechanism for induction of *ERα* expression in granulosa cells appeared to involve tyrosine kinase inhibitory properties, but that indirect effects of genistein on the hypothalamic-pituitary axis could not be ruled out. In contrast, the study authors concluded that increases in multi-oocyte follicle numbers require a functional ERβ.

Strengths/Weaknesses: Strengths of the study are use of an adequate number of animals/group and multiple dose levels, including some relevant to human exposure; however the sc dose route is a weakness. The experimental design was appropriate for determining mechanisms of effect by comparing results of genistein to those of other tyrosine kinase inhibitors and using ERα and ERβ knock-out mice to examine estrogenicity of genistein. A weakness of the study was no examination of animals and tissues after PND 19. Study of adult animals would have been useful. The materials and methods section did not state what statistical methods were used in Table 1 of the publication. Dunnett's test was used to compare each of the exposure groups against control. One of the exposure groups was statistically significantly different from control. However, the remaining two exposure groups that were not statistically significantly different from control were obviously statistically significantly different than the other exposure group. However, this result is not addressed.

Utility (Adequacy) for CERHR Evaluation Process: Results of this important paper suggest that neonatal exposure of female mice can trigger deleterious effects in maturing ovaries and pinpoint ERs and tyrosine kinase as molecular targets. The study is of limited utility in the evaluation because the study is well-designed and conducted, but the sole endpoint measured is of unknown relevance to effects on reproduction in humans.

Jefferson et al., 2005 ***(592)***, from NIEHS, examined the effects of neonatal sc injection treatment with genistein exposure on the reproductive systems of female mice. CD-1 mice used in this study were fed NIH-31 laboratory chow, a feed containing low levels of phytoestrogens (~98 µg/g genistein and daidzein, equivalent to an intake of ~16.7 mg/kg bw/day). Standardized litters of 8 female pups were created using randomly assigned pups from at least 3 litters. On PND 1–5 **[day of birth not defined]**, pups were given genistein 0.5, 5, or 50 mg/kg bw/day **[purity not stated]** in corn oil by sc injection. **[Controls were said to be untreated.]** Authors stated that the doses represented ranges of exposure in pregnant and lactating vegetarian mothers and in infants fed soy-based formulas. **[The Expert Panel noted the study of Doerge et al., 2002** ***(216)*****, summarized in Chapter 2 in which mouse neonates given genistein 50 mg/kg bw/day sc on PND 1–5 had C_{max} blood values for genistein aglycone of 1.4–2.3 µM and C_{max} values for conjugated genistein of 3–5 µM. These values correspond to 378–621 µg/L for the aglycone and 810–1350 µg/L genistein equivalents for the conjugates. As noted in Chapter 1, mean plasma genistein (aglycone + conjugates) in human infants on soy formula was 1455.1 ng/ml at the 75th percentile** ***(94)*****. Pregnant women at term had plasma genistein (aglycone + conjugates) levels up to 303 nM or about 82 µg/L** ***(97)*****. Vegetarian and Japanese women had plasma genistein (aglycone + conjugates) levels of about 17–502 nM, or 4.6–136 µg/L genistein equivalents.]**

Mice were examined for vaginal opening (n=15 or 16/group) and monitored for estrous cyclicity (n=8/group) over a 2-week period at 2 and 6 months of age. At 2 months of age in all dose groups, and at 4 and 6 months of age in the lower 2 dose groups, 8 mice/group were mated to untreated males for 2 weeks or until a vaginal plug was detected. The same mice were used for each mating period. Mice were allowed

to litter and pups were sexed and counted. Ovaries from 5–8 mice/group were collected, and corpora lutea were examined at 6 weeks and 4 months of age. Ovulatory capacity was examined at 4 months of age in 14–16 mice/group by counting oocytes following treatment with human chorionic gonadotropin. Serum progesterone and 17β-estradiol levels were measured at 19 days of age in 8 mice/group; some pooling of samples was required to obtain enough blood resulting in 2–8 samples/group. Continuous data were analyzed using ANOVA followed by Dunnett's test. Categorical data were analyzed using the Fisher's exact test; pregnancy rates were also analyzed using the Cochran-Armitage linear trend test.

An intense reddening of the vaginal area was observed in mice from the 50 mg/kg bw/day group from weaning through adulthood. Vaginal opening was described as tending to occur earlier in the 0.5 mg/kg bw/day group and later in the 50 mg/kg bw/day group, although mean day of vaginal opening was not significantly affected by treatment. No significant effects on serum progesterone or 17β-estradiol levels on PND 19 were detected. Estrous cyclicity data are summarized in **Table 96**.

Table 96. Estrous Cyclicity Effects in Mice Treated as Neonates with Genistein (Jefferson et al., 2005)

Endpoint	*Genistein, mg/kg bw/day*					
	0	*0.5*[a]	*5*[a]	*50*[a]	*BMD_{10}*[b]	*$BMDL_{10}$*
Evaluated at 2 months of age						
Extended diestrus	0	2	4	0	2	1
Extended estrus	0	1	3	6	9	6
Persistent estrus	0	0	0	1	49	28
Evaluated at 6 months of age						
Extended diestrus	5	5	4	1	Not calculated	
Extended estrus	0	1	2	2	33	14
Persistent estrus	0	0	1	5	17	10

Data shown as number of mice with the indicated effect of a total of 8/group.

[a] The authors state "Differences among the doses in the distribution across categories are highly significant at 2 and 6 mo using the Fisher exact test ($P<0.01$)."

[b] See the footnote to Table 95 for an explanation of the use of benchmark dose in this report. A probit model was used. The 50 mg/kg bw/day dose was omitted for benchmark dose modeling of extended diestrus at 2 months of age.

From Jefferson et al.,2005 *(592)*.

Treatment with genistein resulted in significant and dose-related increases in estrous cycle abnormalities at all dose levels. The effects were more severe at 6 than at 2 months of age. There was an increased incidence of persistent estrus in the high-dose group. Fertility parameters for which there was evidence of dose-related effects are summarized in **Table 97**. No significant effects were observed for number of plug-positive mice at any age. The number of pregnant mice, defined as the number of mice who delivered live pups, was significantly reduced in all dose groups at 2, 4, and 6 months of age. At 2 months, none of the dams in the 50 mg/kg bw/day group gave birth to live pups. A second group treated with 50 mg/kg bw/day on PND 1–5 also failed to deliver live pups; therefore, the 50 mg/kg bw/day dose was not tested at 4 and 6 months of age. In the 0.5 and 5 mg/kg bw/day groups, the reduction in pregnancies was most pronounced at 6 months of age, and the authors stated that the effect was consistent with early reproductive senescence.

Table 97. Fertility Effects in 1st Experiment in Mice Treated with Genistein as Neonates (Jefferson et al., 2005)

Endpoint	Genistein, mg/kg bw/day							
	0	0.5	5	50	BMD_{10}[a]	$BMDL_{10}$	BMD_{1SD}	$BMDL_{1SD}$
Evaluated at 2 months of age								
Number of pregnant/plug-positive	6/6	6/6	6/8[c]	0/16[c]	4	2		
Live pups/dam[b]	15.2 ± 0.8	13.2 ± 0.9	11.5 ± 1.6[d]	0	2	1	4	2
Corpora lutea/dam[b]	N.D.							
Evaluated at 4 months of age								
Number of pregnant/plug-positive	6/6	4/4	7/8	–	5	2		
Live pups/dam[b]	12.8 ± 1.6	12.5 ± 1.6	10.0 ± 2.4[e]	–	2	1	8	3
Corpora lutea/dam[b]	9.2 ± 1.3	13.4 ± 2.0	18.0 ± 1.0	0	44	7	47	21
Evaluated at 6 months of age								
Number of pregnant/plug-positive[d]	7/7	3/5	2/5	–	1	0.6		
Live pups/dam[b]	13.7 ± 1.4	9.3 ± 2.2	8.5 ± 2.5[e]	–	1	0.7	4	2
Corpora lutea/dam[b]	N.D.							

[a] See the footnote to Table 95 for an explanation of the use of benchmark dose in this report. A probit model was used for dichotomous data.
[b] Mean ± SEM
[c] Significantly different from control.
[d] Significant linear negative trend.
[e] Significantly different from control when 3 time points combined.
N.D. = Not determined.
From Jefferson et al., 2005 *(592)*.

Number of live pups did not differ significantly when each time period was analyzed separately. However, when all time periods were analyzed together, there was a significant reduction in live pups in the 5 mg/kg bw/day group. Number of corpora lutea was not affected by genistein treatment at 6 weeks of age. At 4 months of age, mice in the 5 mg/kg bw/day group had significantly more corpora lutea, but none were observed in mice of the 50 mg/kg bw/day group. No significant difference was detected in number of ovulated oocytes following treatment of mice with human chorionic gonadotropin at 4 months of age.

An additional study was conducted to further assess implantation defects and pregnancy loss in mice treated with 50 mg/kg bw/day genistein. Female mice treated with genistein 0 or 50 mg/kg bw/day (n=64/group) were mated at 2 months of age to untreated males. Reproductive tracts were collected from half the plug-positive mice on GD 6, 8, or 10 (GD 0=plug) for an examination of implantation and resorption sites. Blood was collected from the other half of the plug-positive mice on GD 6, 8, or 10 and from non-pregnant mice (n=3–7 group) to measure serum levels of progesterone, 17β-estradiol, and testosterone. Ovaries were collected at each time point for an examination of corpora lutea in 3 sections/ovary.

Fertility parameters in mice treated with genistein 50 mg/kg bw/day are summarized in **Table 98**. No significant treatment effect on the number of plug-positive mice following mating were detected. Genistein treatment resulted in significant reductions in the percentage of pregnant mice, the number of mice with visible implantation sites, and the number of implantation sites. In addition, implantation sites in genistein-treated mice were smaller than in controls. The number of corpora lutea was reduced by genistein treatment in pregnant mice (**Table 98**) and was even lower in non-pregnant mice (n=~1–3 on study days 6, 8, and 10). In pregnant mice, no significant overall treatment effects on serum progesterone, 17β-estradiol, or testosterone levels were detected, although genistein treatment was associated with a **[~90%]** decrease in serum progesterone on days 6 and 8 and a **[~83%]** decrease in serum testosterone on day 8.

Table 98. Fertility Effects in 2nd Experiment of Mice Treated with Genistein as Neonates (Jefferson et al., 2005)

Endpoint	*Evaluation Day*	*Dose, mg/kg bw/day*	
		0	*50*
Number (%) mice with implantation sites	GD 6	16/18 (89%)	8/13 (62%)*
	GD 8	18/19 (95%)	7/19 (37%)*
	GD 10	6/6 (100%)	5/11 (45%)*
Number of implantation sites/mouse (estimated from a graph)	GD 6	**[14]**	**[8]***
	GD 8	**[14]**	**[11]***
	GD 10	**[12]**	**[5]***
% Pregnant mice (estimated from a graph)	GD 6	**[90%]**	**[65%]**
	GD 8	**[95%]**	**[35%]***
	GD 10	**[100%]**	**[40%]***
Number of corpora lutea (estimated from a graph)	GD 6	**[23]**	**[6]***
	GD 8	**[18]**	**[8]***
	GD 10	**[13]**	**[9]**

*Statistically significant compared to controls.
From Jefferson et al., 2005 *(592)*.

The study authors concluded that treatment of neonatal mice with environmentally relevant doses of genistein resulted in abnormal estrous cycles, altered ovarian function, early reproductive senescence, and subfertility or infertility.

Strengths/Weaknesses: A strength of this study is an adequate number of animals used/group. Administration of genistein by sc injection provided clear information on doses received by animals but is not a route of exposure relevant to humans. A wide range of genistein doses was administered at levels relevant to human exposure. The exposure period (PND 1–5) was well-defined. A variety of endpoints, including hormonal status, was examined. A weakness of the study is that assigning ovarian senescence without histological confirmation is not conclusive. This is one of the few studies whereby the numbers of animals for each of the endpoints assessed is described in the manuscript. This allows one to assess the strength of the statistically significant findings or determine the strength of the findings via the graphics presented in the manuscript.

Utility (Adequacy) for CERHR Evaluation Process: This study is of high utility for the evaluation process. It is a well-designed, i.e., used an appropriate number of animals and relevant dose levels, and very important study that highlights long-term effects of neonatal exposure to genistein on a number of complementary endpoints in the female reproductive system, including prolonged estrous cycles, altered ovarian function, subfertility, and early reproductive senescence. It also shows that a relatively low genistein dose of 0.5 mg/kg bw/day has deleterious consequences.

Jefferson et al., 2006 ***(593)*** supported by NIEHS, further evaluated the production of multi-ovarian follicles seen after neonatal sc injection treatment with genistein treatment in their previous study *(591)*. Female CD-1 mouse neonates were pooled and randomly assigned to dams as all-female litters of 8 pups. On PND 1–5 **[day of birth not indicated]**, pups were treated with genistein **[purity not given]** 50 mg/kg bw/day. Control pups were not treated. Pups were decapitated on PND 2, 3, 4, 5, or 6 (8 mice/treatment group/age) and ovaries were fixed in paraformaldehyde. Whole ovaries were labeled with Stat3, a germ cell marker. The number of individual oocytes relative to the number of oocytes in nests was determined by confocal microscopy of 2 regions per ovary. Four sections at least 20 μm apart were evaluated for proportion of follicle types (primordial, primary, secondary) based on morphologic criteria. Transmission electron microscopy was used to evaluate ovaries from PND 4 mice for the presence of intracellular bridges connecting oocytes. Immunohistochemistry staining for poly (adenosine diphosphate-ribose) polymerase 1 and terminal deoxynucleotidal transferase-mediated dUTP nick-end labeling (TUNEL) were used to assess apoptosis. Data were analyzed using 2-way ANOVA with treatment and day as main effects.

The percentage of unassembled follicles, defined as follicles in which the oocytes were not completely surrounded by granulosa cells, was increased in genistein-treated mice (73.4 ± 3.7%, mean ± SEM) on PND 4 compared to untreated controls (56.7 ± 2.9%). The percentages of primordial and primary oocytes were correspondingly decreased by genistein treatment. In control sections, 44% of oocytes were single, compared to 21.2% of oocytes in sections from genistein-treated mice, with large oocyte nests still apparent in the genistein-exposed ovaries. A significant difference in percentage and number of single oocytes between control and genistein-exposed ovaries was identified on PND 4–6. On PND 4, there were no intracellular bridges among 325 oocytes from control animals and there were 3 intracellular bridges among 633 oocytes from genistein-treated animals. Counts on PND 2, 4, and 6

showed a larger number of oocytes in sections from genistein-exposed ovaries on PND 4 and 6 than in sections from control ovaries. Follicle counts per section on PND 4 were 58 in control ovaries and 79 in genistein-exposed ovaries. Follicle counts per section on PND 6 were 41 in control ovaries and 52 in genistein-exposed ovaries. There were no detected differences in ovary size that would explain the differences in follicle counts per ovarian section. **[Follicle counts were estimated from a graph; ovarian size data were not shown.]** The percentage of cells positive for apoptosis markers was decreased on PND 3 in sections from genistein-treated mice compared to controls. TUNEL staining was increased in genistein-exposed ovaries compared to controls on PND 2, but poly (adenosine diphosphate-ribose) polymerase 1 staining differences by treatment on this day were not detected.

The authors concluded that neonatal genistein treatment in mice resulted in an increase in multi-oocyte follicles and fewer single oocytes as a result of incomplete breakdown of oocyte nests. There were also deficits in programmed cell death, which normally reduces the number of oocytes by two-thirds. The larger number of oocytes in the ovary of genistein-treated mice would provide pre-granulosa cells with a larger number of oocytes to be surrounded, and an increase in unassembled follicles was identified in ovaries from genistein-treated mice. The authors cited other authors' work using neonatal treatment with diethylstilbestrol and their own previous work with genistein *(591)* as supporting the hypothesis that the interference of genistein with ovarian differentiation was a function of the compound's estrogenic activity.

Strengths/Weaknesses: This is a well-designed study that provides further characterization for a well-studied phenomenon. Strengths of this study are adequate numbers of animals and the exposure time-frame. Several ovarian parameters were examined. A weakness of this study is that the effects of only 1 high dose level were examined and that genistein was given by sc injection. The author's conclusion that the effects of genistein on ovarian differentiation were due to its estrogenic activity would have been strengthened by use of a positive control.

Utility (Adequacy) for CERHR Evaluation Process: Although only one dose was examined in this study, previous work by the same authors examined dose-response effects in the ovary following neonatal exposure. This study provides additional information by looking more closely at ovarian development and apoptosis and proposes a potential mechanism for multi-oocyte follicles. This study is of limited utility for the evaluation because it was very well-designed and conducted.

Jefferson et al., 2009 ***(594)*****,** supported by the Intramural Research Program of the NIEHS examined oocyte developmental competence and timing of embryo loss in neonatal mice injected subcutaneously with genistein. Pregnant CD-1 (Crl: CD-1 [ICR] BR) mice were obtained from the NIEHS (Research Triangle Park, NC) fed NIH-31 diet and allowed to deliver their litters **[bedding not described]**. Female pups were administered subcutaneous injections on PND 1 through PND 5 of genistein suspended in corn oil (50 mg/kg bw/day, 98% pure) or corn oil alone (control) **[basis of dose selection not stated]**; pups were weaned on PND 22 and raised for use on the following six experiments: <u>*Experiment #1*</u>: Ovulation was induced and the eggs were collected and examined for their general morphology and spindle structure (n=8 for genistein, n=6 for control). *In vitro* fertilization of cumulus-enclosed eggs was performed and the fertilized eggs were cultured to the oocyst stage of development. <u>*Experiment #2*</u>: One-cell embryos (two pro-nuclei stage) were collected from the oviducts of genistein-treated and control females and cultured to the blastocyst stage. The resulting blastocysts were transferred to

untreated control pseudopregnant recipients. Recipients **[number not stated]** received 16 blastocysts (8 per uterine horn) from one treatment group and allowed to deliver their pups. Ten female pups from each group were bred at 8 weeks of age to determine their fertility. *Experiment #3*: Eggs were collected from superovulated genistein-treated and control females 17-18 or 21-22 hours after hCG administration and placed in culture **[number of females used not stated]**. *Experiment #4*: One-cell embryos collected 21-22 hours after hCG administration **[number of females used not stated]** were monitored daily to determine developmental progression and the average percentage of embryos reaching each stage was determined. *Experiment #5*: Embryos were flushed from the oviduct or uterus of vaginal plug-positive females at 24, 48, 72, or 92 hours after hCG administration **[number of females used not stated]**. Embryos were subsequently cultured *in vitro* to determine progression to the blastocysts stage. *Experiment #6*: Blastocysts were collected from untreated control females and transferred to control or genistein-treated pseudopregnant recipients (n=9 for genistein, n=5 for control). Control blastocysts (n=7 or 8) were transferred into a single uterine horn of each recipient and the uterus was examined 8 days following transfer for the presence of implantation sites. *All experiments*: Statistical significance was determined using nonparametric Wilcoxon or Mann-Whitney *U*-test or Fisher's exact test as appropriate.

Experiment #1: Genistein-treated females ovulated a mean of 29.6 eggs, the control females ovulated a mean of 28.8 eggs; the appearance of the ovulated eggs in both groups was similar. When spindles of the eggs were stained for α-tubulin and DNA, the spindle morphology was normal in almost all cases. *In vitro* fertilization resulted in efficient fertilization in both groups and there was no difference between the treatment groups in the timing of development or the percentage of embryos that reached the blastocyst stage. *Experiment #2*: All recipients delivered pups and there were similar numbers of live pups per litter in both groups; all pups were apparently healthy and survived to weaning. All plug-positive F_1 females (n=9 for genistein, n=8 for control) delivered their litters; litter size was 14.4 for the genistein group and 15.0 for the control group. *Experiment #3*: By 17-18 hours after hCG administration, there were fewer eggs fertilized in the genistein-treated group (36%) compared to controls (89%); however, by 21-22 hours after hCG administration, most eggs were fertilized in both groups (98% for genistein-treated females, 92% for control females). *Experiment #4*: There was no difference in the ability to progress to the two-cell stage in embryos from genistein-treated vs. control mice. However, with additional time in culture, slightly fewer embryos from genistein-treated females progressed to the subsequent preimplantation embryo stages, this was statistically significant at the blastocysts stage. Embryos from genistein-treated mice reached cleavage stages beyond the four-cell stage more slowly than control. By 72 hours after hCG administration, control embryos were split evenly between four-cell and five- to eight-cell stages of development, while embryos from genistein-treated females were predominately at the four-cell stage with only a few at the five- to eight-cell stages of development. *Experiment #5*: The mean numbers of embryos per mouse collected at 24 and 48 hours after hCG administration were not different between groups; however, after 72 and 92 hours after hCG administration, there was a significant reduction in the number of embryos retrieved, with about 50% fewer embryos retrieved from genistein-treated mice than controls at both time points. Embryos collected 24 hours after hCG administration showed a slight reduction in blastocysts development, while most embryos retrieved 48, 72, and 92 hours after hCG administration progressed to the blastocysts stage. *Experiment #6*: Three of the 5 control recipients had normal-size implantation sites (the other two were not pregnant), only two of the nine genistein-treated recipients had any implantation sites, and these few sites were obviously smaller (less than half the size) than implantation sites in the control group.

Authors' conclusion: The results suggest that oocytes from mice treated neonatally with genistein are developmentally competent; however oviductal environment and the uterus have abnormalities that contribute to the observed reproductive failure.

Strengths/Weaknesses: Strengths of the study are that many end points in establishment of pregnancy were evaluated. The findings of effects in adult animals following a limited period of dosing during neonatal life demonstrate an imprinting and potential epigenetic effect. Weaknesses include use of a sc injection route of administration, relatively high dose level, and a lack of description of the numbers of females used in all experiments. The statistical analyses of the data presented are quite simplistic. Figure 2A presents a graph of embryo progression during 4 days *in vitro*. The analysis compares the percentage of embryos that have progressed to each stage for each stage individually (thereby assuming that the data points are independent of each other during progression). It would have been better to analyze the data via survival analysis methods, i.e., Kaplan-Meier survival functions with an accompanying log-rank test to compare the survival distributions of embryos between control and genistein.

Utility (Adequacy) for CERHR Evaluation Process: This study is of limited utility for the evaluation process due to use of sc injection as the route of administration. The study presents a multi-faceted approach for observing numerous reproductive end points and attempt to determine the mechanistic underpinnings of potential effects.

Newbold et al., 2001 ***(334)***, from NIEHS, examined the effects of neonatal sc injection treatment with genistein on the development of uterine adenocarcinoma in mice. Pregnant CD-1 mice were fed an NIH 31 mouse chow containing a low concentration of genistein (46 µg/g feed). At birth, all litters were pooled and standardized to 8 female pups/dam. An estrogenicity study was conducted in one group of pups and is described in of Chapter 2. On PND 1–5 **[day of birth not specified]**, 13–17 pups/group were sc injected with corn oil or 50 mg/kg bw/day genistein. The dose was said to be less than an order of magnitude higher than genistein exposures in infants receiving soy formula. A 0.001 mg/kg bw/day dose of diethylstilbestrol was used as a positive control. Mice were killed at 18 months for histopathologic examination of reproductive organs. Reproductive lesions observed at a greater incidence in the genistein compared to the control group are summarized in **Table 99**. Genistein treatment increased the incidence of benign and malignant lesions. Adenocarcinoma was the most notable lesion observed in the genistein group and the study authors noted that similar malignant lesions were never observed in control mice in their laboratory. Based on the findings of this study, the study authors expressed concern about use of infant soy formula.

Table 99. Reproductive Lesions Occurring in Mice Treated with Genistein (Newbold et al., 2001)

Lesion	*Incidence of Lesion (%)*		
	Control	*Genistein*	*Diethylstilbestrol*
No corpora lutea	0/13 (0)	17/17 (100)	4/12 (33)
Abnormal oviduct histology	0/13 (0)	14/14 (100)	5/10 (50)
Uterine squamous metaplasia	Not stated	11/17 (64)	5/13 (38)
Cystic endometrial hyperplasia	3/16 (19)	8/17 (47)	7/13 (54)
Uterine adenocarcinoma	0/16 (0)	6/17 (35)	4/13 (31)

Treatment PND 1 – 5 with SC vehicle, 50 mg/kg bw/day genistein, or 1 µg/kg bw/day diethylstilbestrol.
From Newbold et al., 2001 *(334)*.

Strengths/Weaknesses: Strengths of the study were use of an adequate number of mice/group and comparison with diethylstilbestrol. However, estrogenic activity observed in *in vitro* transcription/binding studies may not reflect physiological interactions and effects. A weakness of the study was the use of only 1 high genistein dose, which exceeded reported exposures in infants fed soy-formula, and the sc route of administration. A minimum of 8 pups per treatment was included in each group. Uterine weight:body weight ratio means and their accompanying standard errors (SEs) are reported in Table 1 of the publication, If the sample sizes are equal or roughly equal between the DES and genistein exposure groups, then one could attribute the larger SE of the genistein group to variability observed in response. However, if the sample size for the genistein group is considerably smaller than the DES group, then the larger SE could be partially attributed to the smaller sample size. Without information on sample size per group, it is difficult to assess the results presented in Table 1.

Utility (Adequacy) for CERHR Evaluation Process: The study is of limited utility for the evaluation process due to the route of administration. The uterine lesion findings in particular are intriguing and potentially very important and highlight the need for additional research and confirmation on the long-term effects in the uterus following short-term exposure to genistein early in life.

Nikaido et al., 2005 ***(595),*** in a study supported by the Japanese Ministry of Health, Labor, and Welfare, examined the effects of prepubertal genistein exposure via sc injection on endocrine-sensitive tissues in female mice. At 15 days of age, 17–24 female CD-1 mice were sc injected with 0 (DMSO vehicle) or 10 mg/kg bw/day genistein (≥99% purity) for 4 days. Body weights were measured weekly. All mice were monitored for vaginal opening. Vaginal smears were taken for 21 days during 3 time periods beginning at 5, 9, and 21 weeks of age. Six mice/group were killed and necropsied at 4, 8, 12, and 24 weeks of age. Ovary, uterus, vagina, and mammary gland were examined histologically. Data were analyzed by ANOVA parametric test, Kruskal-Wallis non-parametric test, and/or Fisher's protected Least Significant Difference test.

No genistein effect on body weight was detected. Vaginal opening was accelerated by 3.1 days in the genistein-treated mice. No effect of genistein treatment on estrous cycles was observed. At 4 weeks of age, 2/6 control mice and 3/6 genistein-treated mice had no corpora lutea. No effects on corpora lutea were noted in mice killed at later periods. No polyovular ovarian follicles or morphological abnormalities in vaginal or uterine epithelium were observed. Genistein treatment was not observed to affect mammary gland development. Other possibly estrogenic substances were also examined, and it was reported that zearalenone, zeranol, and diethylstilbestrol also accelerated vaginal opening in addition to disrupting estrous cycles. The study authors concluded that prepubertal genistein treatment accelerated vaginal opening in mice.

Strengths/Weaknesses: The use of very pure genistein, low-phytoestrogen chow, and diethylstilbestrol as a positive control are strengths of this study. Weaknesses include the use of only 1 genistein dose level, the sc injection route of administration, and the small number of animals evaluated at each time point.

Utility (Adequacy) for CERHR Evaluation Process: This study is of limited utility in the evaluation process. It provides confirmation of the ability of genistein to decrease age of first vaginal opening.

Tang et al., 2008 ***(596)***, supported by the Intramural Research Program of the NIEHS and the Department of Defense Prostate Cancer Program, sought to discover novel uterine genes whose expression is altered by an epigenetic mechanism in neonatal mice exposed to diethylstilbestrol or genistein by subcutaneous

injection. Adult CD-1 mice [Crl:CD-1(ICR)BR] were obtained from Charles River Breeding Laboratories (Raleigh, NC) and fed NIH-31 laboratory mouse chow that had been tested for estrogenic activity and found to be negative. Mice were mated and allowed to deliver **[bedding not described]**; at birth, litters were standardized to 8 female pups per litter and assigned to one of five treatment groups. Female pups were treated on days 1-5 with 1 (DES 1) or 1000 (DES 1K) μg/kg/day of diethylstilbestrol, 50 mg/kg bw/day (GEN 50K) of genistein dissolved in corn oil, or with corn oil alone (oil-treated controls) by subcutaneous injection (n=42/group) **[basis of dose selection not stated, purity not stated for any substance]**. For each treatment group, 18 pups were killed at 19 days of age (before puberty). Three uteri were pooled to generate a total of six Day 19 samples per group. The remaining 24 pups per group were either ovariectomized (OVX, n=12/group) before puberty or left intact (n=12/group). Six OVX and six intact mice from each group were killed at 6 months of age and the remaining mice were killed at 18 months of age. Uteri were snap-frozen for DNA and RNA isolation. Methylation-sensitive restriction fingerprinting (MSRF) was performed, real-time RT-PCR was conducted and *Nsbp*1 levels were determined, and bisulfite genomic sequencing was performed. All data groups were analyzed by one-way ANOVA followed by post hoc Bonferroni tests with two-tailed distribution. Unsupervised two-way hierarchical clustering of methylation profiles was performed and the statistical significance was assessed with Fisher's exact test for the 2-by-2 table.

Neonatal exposure to diethylstilbestrol or genistein induced permanent alterations in DNA methylation status of specific genes; *Nsbp*1 was selected as the target gene because its gene product binds to nucleosome core element and may play critical roles in chromatin remodeling. In the absence of adult ovarian steroids (OVX females), neonatal treatment with diethylstilbestrol/genistein exerted differential effects on alterations in gene expression/methylation status of *Nsbp*1 in an age-dependant manner. There were no significant differences between the oil-treated controls and the two diethylstilbestrol-treatment groups; animals treated with GEN 50K showed distinct hypermethylation in this *Nsbp*1 promoter CGI (CpG island) as compared to the other three groups. In OVX mice, the neonatal genistein effects persisted and methylation increased progressively throughout life. In the oil-treated controls, no drastic changes in the extent of methylation were found throughout life; however, the mice in the DES 1 group exhibited a moderate increase in *Nsbp*1 promoter CGI methylation as they aged and mice in the DES 1K group consistently displayed the lowest degree of methylation. In 18-month-old mice, a small but significantly greater expression of *Nsbp*1 was observed in the oil-treated animals whereas a mild decline of gene expression was seen in the DES 1 and GEN 50K groups. The most dramatic effect was observed in the DES 1K group that displayed chronic overexpression (>4-fold) of transcript levels of *Nsbp*1 throughout life. In the presence of adult ovarian steroids (intact females), neonatal treatment with diethylstilbestrol/genistein exerted similar effects on the alteration of gene expression/methylation status of *Nsbp*1 in a manner independent of the neonatal treatment regimen.

Authors' conclusion: The life reprogramming of uterine *Nsbp*1 expression by neonatal diethylstilbestrol/genistein exposure appears to be mediated by an epigenetic mechanism that interacts with ovarian hormones in adulthood.

Strengths/Weaknesses: **weaknesses:** Strengths of the study include use of DES as a positive control, appropriate statistical power, control for litter effects, and mechanistic data. Weaknesses include use of sc injection route of administration. The interpretation is complicated by opposite outcomes following treatment with DES versus genistein. Also, although changes in DNA methylation status were seen, translation to physical outcome is not clear.

Utility (Adequacy) for CERHR Evaluation Process: This study is of limited utility in the evaluation process due to inability to assign adequate relevance of findings to human risk

3.3.1.1.6 Postnatal - Male Mice (oral)

Jung et al., 2004 ***(597)***, supported by the Korean Ministry of Health and Welfare, examined reproductive development in male mice orally-treated with genistein following weaning. ICR mice used in this experiment were obtained from dams that were fed a soy-based Purina chow diet during gestation and lactation. **[The number of dams and distribution of pups were not specified.]** Male mice were weaned and fed AIN-76A, a casein-based diet, beginning on PND 21. The mice were divided into groups of 10 and gavaged for 5 weeks with genistein (>98% purity) in corn oil at 0 or 2.5 mg/kg bw/day or 17β-estradiol 7.5 μg/kg bw/day. Following treatment, animals were killed, and testis, epididymis, and prostate were removed and weighed. Sperm count and motility were determined. Reproductive organs were fixed in Bouin fluid, and histopathologic evaluation was conducted. Total RNA was isolated from the reproductive organs to measure expression of phospholipid hydroxide glutathione peroxidase. Data were evaluated by ANOVA and Least Significant Difference testing.

No significant effect of genistein on body weight gain or relative weights of testis, epididymis, or prostate were detected. **[Absolute organ weights were not reported.]** A significant decrease in prostate weight was observed in mice treated with 17β-estradiol. There was no detected reduction in testicular sperm count after treatment with genistein or 17β-estradiol, but 17β-estradiol significantly reduced epididymal sperm count. Although no significant effects on sperm motility parameters were detected, the study authors stated that motility was slightly higher in the genistein-treated mice and slightly lower in the 17β-estradiol-treated mice. Expression of phospholipid hydroxide glutathione peroxidase was significantly higher in testis and prostate of mice treated with genistein and 17β-estradiol **[~2-fold higher in testis and 1.5-fold higher in prostate of genistein-treated compared to control mice]**. No pathologic lesions were observed in the testis, epididymis, or prostate of genistein-treated mice **[data were not shown]**. In contrast, 17β-estradiol treatment induced lesions in testicular germ cells, epididymis, and prostate. The study authors concluded that these results suggested that genistein intake had no observable adverse effect on the development of the reproductive system in mice.

Strengths/Weaknesses: A strength of this study is use of an adequate number of animals. Oral administration mimicked human exposure, and gavage treatment permitted determination of doses administered. Other strengths included the long-term exposure (5 weeks), comparison with 17β-estradiol, and examination of multiple endpoints. A weakness of the study is that only one dose level was tested. The broad exposure time-frame extending from prepuberty into beginning of adulthood increased complexity of data interpretation, compared to a more limited exposure time-frame; however, there were not many effects to analyze.

Utility (Adequacy) for CERHR Evaluation Process: This study is of no utility because dosing began on PND 21, after the most relevant lifestage for an assessment of soy infant formula.

Lee et al., 2004 ***(598)***, supported by the Ministry of Health and Welfare, Republic of Korea, examined the effects of oral treatment with genistein prior to and during puberty on reproductive development in male ICR mice. After being weaned to a casein-based diet (which was used in dams as well) on PND 21, mice were treated orally with genistein (>98% purity) in corn oil at 0, 2.5, or 5.0 mg/kg

bw/day for 5 weeks (n=10/ group). A positive control group was given 17β-estradiol. **[Gavage and daily treatment are assumed.]** At the end of the 5-week treatment period, reproductive organs were removed. Differences in weight and histopathology of reproductive organs, sperm count and motility, and levels of phospholipid hydroxide glutathione peroxidase mRNA expression were evaluated. Sperm count was obtained using a hemocytometer after homogenization of testicular parenchyma and cauda epididymis tissue. Cauda epididymis was placed in modified Tyrode medium supplemented with bovine serum albumin, and the sperm suspension was collected. Computer-assisted sperm analysis (CASA) was performed. Total RNA was extracted from the testis, epididymis, and prostate and evaluated using RT-PCR. Data were analyzed by ANOVA.

No significant differences in body or organ weights between the groups were detected with the exception of lower body and epididymis weight in the 17β-estradiol treatment group. 17β-Estradiol treatment also decreased sperm count and motility. Slight decreases in sperm counts did not achieve statistical significance in the genistein-treated groups. Although differences in sperm motility parameters were not significant, many motility characteristics were said to have been increased by exposure to genistein. The genistein groups were also found to have a dose-dependent increase in the expression of phospholipid hydroxide glutathione peroxidase mRNA in the testis, epididymis, and prostate. The 17β-estradiol group also had significantly greater expression of phospholipid hydroxide glutathione peroxidase mRNA in all three organs. Histopathology exams in both genistein dose groups showed hyperplasia of Leydig cells in the testis and an increase of interstitial fibroblasts and slightly irregular arrangement of the epithelium in the epididymis. The 17β-estradiol group was found to have severe damage of the testis and epididymis.

The study authors concluded that slight decreases in sperm counts and improvement of sperm motion quality following dietary genistein intake by juvenile mice suggest that genistein may affect reproductive development in males.

Strengths/Weaknesses: Strengths of the study include adequate numbers of animals, relevant doses, examination of multiple endpoints, and comparison with 17β-estradiol.

Utility (Adequacy) for CERHR Evaluation Process: This study is of no utility because dosing began on PND 21, after the most relevant lifestage for an assessment of soy infant formula.

Matrone et al., 1956 ***(599)***, supported by the Tennessee Valley Authority, fed diets containing genistin or diethylstilbestrol to male mice **[strain not given]** beginning at approximately 3 weeks of age. The full dose of genistin or diethylstilbestrol was given in 1 g of a basal diet each day following which untreated basal diet was given ad libitum for the rest of the day. The basal diet contained casein, corn starch, vegetable oil, minerals, cellulose, and cod liver oil. The diet was given for 6 weeks following which the mice were weighed and histologic evaluation performed on testes, adrenal glands, spleen, and kidney. Genistin dose levels (n=10/group) were 0, 9, 13, 36, and 72 mg/day **[0, 439, 833, 3000, and 7200 mg/kg bw/day based on final body weight]** and diethylstilbestrol dose levels were 0.04, 0.08, 0.16, 0.32, and 0.64 µg/day **[1.7, 3.3, 6.6, 14.2, and 30.5 µg/kg bw/day based on final body weight]**. Statistical methods were not discussed. Four mice in the highest-dose genistin group and 2 mice in the second-highest dose genistin group died. An additional 4 deaths were scattered among the other groups. There was a decrease in body weight gain with increasing genistin dose, and the highest-dose genistin animals lost body weight. All diethylstilbestrol-treated animals gained weight,

although weight gain was reduced at the highest dose level. Testis weight decreased with increasing genistin dose from a control weight of 163.4 mg to a weight in the high-dose group of 16.4 mg. Testis weight decreased to a lesser extent with diethylstilbestrol. Histologic evaluation of the testis showed no spermatozoa at the 2 highest genistin dose levels. Spermatozoa were reduced in number in the highest-dose diethylstilbestrol group but were still present. The authors concluded that adverse effects of genistin on survival, growth, and spermatogenesis in mice were due to a mechanism other than estrogenicity inasmuch as diethylstilbestrol did not produce a similar degree of toxicity.

Strengths/Weaknesses: Strengths include the administration of genistin in the diet and the method used to ensure complete intake of the dose. The use of multiple dose levels is also a strength, although the highest dose levels were excessively toxic. Weaknesses include the lack of assessment of the stability of genistin in feed, that lack of evaluation of the basal feed for phytoestrogens, the lack of detail on preparation of tissues for histopathology examination, the failure to report feed consumption, and the failure to report details of the testicular examinations or to include interpretable photographs. The authors' assessment of specific testicular effects of genistin is not reliable given the presence of excessive generalized toxicity.

Utility (Adequacy) for CERHR Evaluation Process: This study is of no utility in the evaluation process.

Montani et al., 2008 *(600)*, supported by the European Union, the Istituto Superiore di Sanità, the Ministero dell'Universit à e della Ricerca Scientifica, and the Fondazione Cariplo, reported a series of experiments designed to characterize genistein-induced ER activation in ERE-tK-LUC transgenic male mice, engineered to express a reporter of ER transcriptional activation. These experiments involved both exposures during development and young adulthood; however, only the experiments involving developmental exposure are described below.

One experiment analyzed the effects of maternal oral gavage treatment with genistein on luciferase activity in the tissues of neonatal transgenic male mice. Lactating mothers were given a single dose of genistein (50 mg/kg) by oral gavage at PND 4 **[purity of the test materials, feed, bedding not described]**. The administered dose produced a serum genistein level of 550 nM. The doses of genistein used were representative of the intake typical of western diets (low intake, from 5 to 50 µg/kg) and eastern diets (high intake, from 50 to 500-1000 µg/kg/day), and the intake of infants fed with soy-based formulas and soy-based nutritional supplements (high intake, from 500 to 5000 µg/kg/day). Heterozygous littermates were screened by PCR analysis for the presence of the transgenic cluster. ERE-tK-LUC sucking pups were killed at 0, 6, 16 and 24 hours after the mother's treatment and luciferase activity was measured in the liver, lung, heart, thymus, testis and brain **[number of animals per group not stated]**. In another experiment, an *in vitro* culture system was used to investigate whether genistein exerts estrogenic effects on the gonads of fetal and developing mice. In this experiment, testes and ovaries obtained from 14.5-day ERE-tK-LUC embryos were cultured and assayed for luciferase activity. Testes and ovaries obtained from 14.5-day CD-1 embryos were cultured and assayed for ^{3}H-thymidine **[number of animals per group not stated]**.

For the enzymatic assay, tissue extracts were prepared, supernatants were collected, and protein concentration was determined by Bradford's assay. Luciferase activity was measured in extracts with a protein concentration of 1 mg/ml, and the light intensity was measured with a lucinometer. In the protein isolation and western blot analysis, tissues were homogenized in lysis buffer supplemented with a protease

inhibitor cocktail. Proteins were separated by electrophoresis and probed with primary antibodies ER-α and ER-β. Immunoglobulins were visualized using an enhanced chemiluminescence detection system. Densitometric quantitation of ERα levels relative to actin levels was performed. Statistical analysis was performed by two-way analysis of variance followed by *post hoc* Bonferroni analysis.

The treatment of nursing mothers on PND 4 resulted in an increased luciferase activity in all pup organs examined; this indicates that genistein passes from the mother's milk at concentrations sufficient to exert estrogenic actions on reproductive and non-reproductive tissues of breast-fed newborns. In the *in vitro* culture system using 14.5-day ERE-tK-LUC embryos, luciferase was induced twofold in the embryonic testes exposed to genistein or estradiol and the addition of ICI-182780 inhibited this response; ovaries did not show any significant response to either compound. Both compounds appeared to stimulate testicular cell proliferation as revealed by a significant twofold increase for ^{3}H-thymidine incorporation in cultured testes, this was inhibited by the antiestrogen ICI-182780. Ovaries were unresponsive to the same treatments.

Author's conclusion: Genistein affects the reproductive and non-reproductive organs of male mice in a dose- and time-dependent manner, at all developmental ages.

Strengths/Weaknesses: Strengths of the study include administration of genistein by oral gavage and measurement of serum genistein concentrations in the dams on PND 4. In addition, the estrogenic response was controlled by using a positive control and an anti-estrogen to block the response. Weaknesses are the lack of a dose-response and validation of the luciferase response in the tissues used. Only a single dose of genistein was administered and the level of serum genistein measured appeared high relative to estimates of human intake. The number of experimental animals per group was not clear and litter effects were not considered. Thymidine incorporation per gonad does not give information as to which cells have incorporated the isotope.

Utility (Adequacy) for CERHR Evaluation Process: The study has limited utility in the evaluation process. The use of increase luciferase activity as an indicator of estrogenic activity and the indirect demonstration of lactational transfer of genistein on PND 4 is of interest. The *in vitro* studies have no utility to the evaluation process.

3.3.1.1.7 Postnatal - Male Mice (non-oral)

Adachi et al., 2004 ***(601)***, supported by the Japanese Ministry of Education, Culture, Sports, Science, and Technology, the Ministry of the Environment, and the New Energy and Industrial Technology Development Organization, evaluated the effect of neonatal genistein treatment by sc injection on testicular gene expression in ICR mice. The animals were injected for 5 days beginning on the day after birth **[injection route not specified]**. Genistein was given in sesame oil at 0 or 1000 μg/mouse/day **[~1000 mg/kg bw/day]**. Diethylstilbestrol 50 μg/mouse/day was injected as a positive control. Animals received a genistein-free diet at weaning. Testes were removed at 12 weeks of age. Some testes were fixed in paraformaldehyde, and some were frozen. Fixed testes were embedded in paraffin and sectioned. Histologic evaluation was performed by light microscopy on hematoxylin and eosin-stained sections, and apoptosis was assessed using TUNEL analysis. Total RNA was extracted from frozen testes. An in-house complementary DNA microarray containing 1754 probes was used to assess gene expression. Real-time RT-PCR was used to evaluate the expression of estrogen and androgen

receptor and to verify the microarray results for two genes that appeared to be down-regulated by genistein and diethylstilbestrol. Body and testis weight data were analyzed using the Student *t* test. Other statistical analyses were not discussed.

No effects of genistein treatment on body weight or on absolute or relative testis weight were detected. Histologic examination and TUNEL staining showed no changes in genistein-exposed animals. The microarray analysis showed little effect on gene expression, except for down-regulation of laminin-γ2 to 57% of control and down-regulation of an expression sequence tag gene to 42% of control. Real-time RT-PCR confirmed these results and showed down-regulation of *ERα* to 42.1% of control and down-regulation of androgen receptor to 49.8% of control. *ERβ* expression was 96.5% of control. Diethylstilbestrol down-regulated the same genes as did genistein. Diethylstilbestrol also decreased body and testis weight and increased TUNEL staining in the testis.

The study authors concluded that neonatal genistein exposure caused changes in testicular gene expression at sexual maturity in spite of a lack of morphologic evidence of injury. They further concluded that the genes identified as having been down-regulated may be markers of neonatal estrogen exposure.

Strengths/Weaknesses: Strengths of this study included an adequate number of animals and time-frame of exposure, examination of several parameters (testis morphometry, apoptosis, gene expression), and comparison with diethylstilbestrol. However, the diethylstilbestrol dose was very high. A weakness is that only one dose level was tested, and that level exceeded environmental relevance. The route of administration (injection) was not relevant to human exposure. The study did not examine fertility of the mice.

Utility (Adequacy) for CERHR Evaluation Process: This study is of limited utility for the evaluation process because a sc route of administration was used and only one dose level was assessed. The findings are reassuring because no effects on testicular morphology or apoptosis at 12 weeks of age following neonatal exposure to a high genistein dose are reported. Gene expression changes could be helpful in identifying molecular targets activated by genistein. *ERα* and androgen receptor expression were suggested as potential biomarkers of genistein exposure.

Shibayama et al., 2001 ***(602)***, supported by the Japanese government and three private foundation grants, evaluated reproductive parameters in male ICR mice after neonatal treatment with genistein by sc injection. Newborn male mice were given genistein **[purity not specified]**, diethylstilbestrol, or the respective vehicles sc each day for 5 days, from the day of birth. There were 8 pups in each treatment group. Genistein doses were 10, 100, or 1000 μg/day **[assuming a 1.4 g bw for an ICR mouse neonate, these doses are 7, 71, and 714 mg/kg bw/day. There was no information on allocation of treatments by litter, culling, weaning, or other details of rearing.]** Animals were killed at 4, 8, or 12 weeks of age. **[The number killed at each time point was not given, but a graph for the 12-week data indicates n=8, suggesting that either there were more than 8 pups/group or that n < 8 at 12 weeks.]** Measured parameters included testis weight, epididymal sperm count, and sperm motility. Quantitative reverse transcription (RT)-PCR of testicular RNA was performed for *ERα* and androgen receptor, using mRNA for glycerol-3-phosphate dehydrogenase as an internal control. ERα protein from testes was quantitated using Western blotting. Statistical methods were not given.

No significant effect of neonatal genistein treatment on testis weight, sperm count, or sperm motility at 12 weeks of age was detected. *ERα* mRNA was described as 20–40% of control levels after neonatal

treatment with genistein 1000 µg/day and 40–80% of control levels **[estimated from a graph]** after the lower doses of genistein. mRNA for androgen receptor **[estimated from a graph]** was 60–80% of control levels after neonatal treatment with genistein 10 µg/day. After the 2 higher doses of genistein, androgen receptor mRNA was about 10% of control at 4 weeks of age, recovering to about 50% of control levels by 12 weeks of age. ERα protein was about 60% of control at 12 weeks of age. **[Statistical testing was not indicated for these data, which were derived from 3 animals per dose group per time point.]** The authors concluded, "These results suggest that estrogenic compounds, even if their activity is not so strong, have long-term effects on the reproductive system at molecular levels." **[The Expert Panel noted that the lack of effect of high doses on sperm count or motility suggests that genistein neonatal exposure does not have deleterious reproductive effects in male mice.]**

Strengths/Weaknesses: A strength of this study is that an adequate number of animals were used. The treatment period (neonatal) was well defined and long-term effects were observed. Results were compared with those of diethylstilbestrol. Long-term effects on *ERα* and androgen receptor expression in testis (they could not detect *ERβ*) were examined in an attempt to identify possible mechanisms. A weakness of the study is that 2/3 dose levels were very high, no environmentally relevant doses were tested, and a sc rout of administration was used. Testis morphology was not examined, despite the availability of samples. There was no mention of the statistical test used.

Utility (Adequacy) for CERHR Evaluation Process: This study is of limited utility for the evaluation process because a subcutaneous route of exposure was used and the dose levels selected were high. Although the doses and route were not relevant to human exposure, the data in this study complement other studies by providing evidence of long-term molecular effects (*ERα* and androgen receptor expression) at the highest dose. The study also provides some insight into potential mechanisms of genistein action.

Strauss et al., 1998 ***(603)***, supported by the European Community, evaluated the effects of neonatal genistein by sc injection on the reproductive tracts of adult male Han-NMRI mice. Mice were "estrogenized" as neonates with sc injections of diethylstilbestrol 2 µg/day, genistein **[purity not specified]** 0.1 or 1 mg/day (~50 or 500 mg/kg bw/day), or corn oil vehicle (controls) on the first 3 days of life (n=10/dose group). Ventral prostates and coagulating glands were dissected and weighed at 3 months of age. Total RNA was extracted from prostatic urethras, and *c-fos* messenger RNA (mRNA) was estimated by Northern blot analysis. In 5 animals/dose group, histologic assessment of urethroprostatic blocks by light microscopy was performed. Statistical analysis was performed using ANOVA followed by Tukey Least Significant Difference test. The study also examined prostatic effects in mice following genistein exposure in adulthood but those studies are not considered relevant to the Expert Panel's evaluation of soy formula.

Genistein treatment did not alter mRNA for *c-fos*. Ventral prostate relative weight was decreased by both genistein dose levels, and coagulating gland relative weight was decreased by the high genistein dose level. Benchmark dose calculations for reproductive organ weights are summarized in **Table 100**. The high genistein dose level produced histologic abnormalities in genital tissues characterized as hyperplasia and disorganization of the epithelium of the prostatic collecting ducts, ventral lobes, and seminal vesicles, with increased fibromuscular stroma and inflammatory cells in the posterior periurethral region. These changes were reported to resemble those produced by diethylstilbestrol treatment. The lower genistein dose level produced hyperplasia in the prostatic collecting ducts in "few animals" **[not otherwise quantified]**.

Table 100. Benchmark Dose Calculations for Treatment-Related Effects on Relative Reproductive Organ Weights of Adult Mice Treated Neonatally with Genistein (Strauss et al., 1998)

Weight	*Benchmark Dose[a], mg/kg bw/day*			
	BMD_{10}	*BMDL*	*$BMD_{1\ SD}$*	*$BMDL_{1\ SD}$*
Body	296	174	326	196
Ventral lobe	154	104	418	261
Coagulating gland	112	94	174	132

[a] See the footnote to Table 95 for an explanation of the use of benchmark dose in this report. A power model was used.
n = 10 pups per dose group.
From Strauss et al., 1998 *(603)*.

The authors concluded that during prostate development, genistein in sufficiently high doses may induce persistent abnormalities similar to those seen with diethylstilbestrol. They remarked that it was not known whether these effects could be produced using dietary phytoestrogens. Further, they observed that the human prostatic development modeled by the neonatal mouse occurs *in utero*, making the mouse model more relevant for maternal dietary exposures during pregnancy than for soy infant formula exposures.

Strengths/Weaknesses: A strength of this study is that adequate numbers of animals were used. The exposure time-frame was well-defined and allowed for a comparison of neonatal and adult sensitivity. Mechanism of action was examined. A weakness of the study is that only high doses were used, although the 50 mg/kg bw/day dose was shown to correspond to circulating genistein levels relevant to human exposure, and the sc route was used. The study was limited in focus because it only examined prostate and no other reproductive tissue.

Utility (Adequacy) for CERHR Evaluation Process: This study is of limited utility for the evaluation process because sc route of exposure was used. This study is useful for studying the balance between beneficial and deleterious effects of genistein exposure on the prostate. The study highlighted differences in prostate sensitivity based on time of exposure. As noted by authors, the neonatal developmental events examined here occur *in utero* in humans; therefore, the neonatal experiments may be more relevant for *in utero* exposure.

3.3.1.2 Mice: Pre- and Postnatal

3.3.1.2.1 Pre- and Postnatal - Male Mice (oral)

Fielden et al., 2003 ***(604)***, supported by the EPA, examined the effects of gestational and lactational oral exposure to genistein on testicular weight and sperm quality in adult mice. Two cohorts of pregnant $B6D2F_1$ mice (n=10–13 per group) were fed AIN-76A, a feed with undetectable levels of isoflavones, throughout pregnancy and lactation. Mice were gavaged with 0, 0.1, 0.5, 2.5, or 10 mg/kg bw/day genistein (98% purity) in corn oil on GD 12 through PND 20, excluding the day of parturition. The lower two doses represented human dietary exposure levels, while the 2 highest doses were selected to replicate potential higher human exposures resulting from dietary supplement intake. The study authors noted that serum genistein levels would likely be higher in humans exposed to the same dose levels. **[This statement appears to have been based on the observation that genistein blood levels in neonatal mice given genistein 50 mg/kg bw/day in 1 study *(216)* were similar to**

levels measured in another study *(84)* in human infants with estimated genistein intakes of 4 mg/kg bw/day from soy formula.] Litter size and weight were evaluated, and anogenital distance was measured on PND 7 and 21. Pups were weaned on PND 21 and fed the AIN-76A diet. On PND 21, one male pup per litter was necropsied. The remaining male pups were killed on PND 105 or 315 for an assessment of testis and seminal vesicle weight, sperm count and motility, and *in vitro* fertilizing ability of sperm. Testicular RNA was isolated from high-dose mice of each age group for an evaluation of gene expression using polymerase chain reaction (PCR). The litter was considered the experimental unit in statistical analyses that included the Shapiro-Wilk test, ANOVA, analysis of covariance (ANCOVA), Kruskal-Wallis test, and Dunnett's test.

No significant effects of genistein treatment were detected on dams giving birth to live pups, pup survival to PND 4 or 21, litter size, pup or litter weight, and sex ratio of pups **[data were not shown]**. A small but significant decrease in anogenital distance (<5%) was observed in the 10 mg/kg bw/day group on PND 21 but not on PND 7 **[data were not shown]**. No significant adverse effects were detected on sperm count or motility or on seminal vesicle, testis, or body weight **[data were not shown]**. Exposure to 10 mg/kg bw/day significantly increased percent *in vitro* fertilization of sperm by 17–18% on PND 105 and 315. Percentages of fragmented eggs were significantly reduced in the 0.1 and 2.5 mg/kg bw/day groups on PND 105 but were statistically increased in the 10 mg/kg bw/day group on PND 315. Significant reductions in percentages of 1-cell fertilized eggs were observed in 315-day-old mice exposed to ≥0.5 mg/kg bw/day genistein. **[Dose-response relationships were questionable for all *in vitro* fertilization parameters.]** Exposure to 10 mg/kg bw/day genistein was not shown to significantly affect the expression of numerous genes, including estrogen and androgen receptors, which were affected in previous diethylstilbestrol studies. The study authors concluded that developmental genistein exposure did not adversely affect sperm quality. **[The Expert Panel noted that the positive effect observed on sperm fertilizing ability is puzzling and could suggest a potential ERβ-mediated role in sperm maturation.]**

Strengths/Weaknesses: Strengths of this study include adequate numbers of animals tested per condition and an adequate dose range (4 doses), including some with relevance to human exposure. Exposure by gavage insured reliable dosing. Molecular parameters (ER, androgen receptor, CYP) were examined. Effects of genistein were compared with those of diethylstilbestrol, although the comparison was made in a previous study and not shown here. A weakness of the study is that data were only provided for a limited number of the endpoints that were examined (*in vitro* fertility and expression of few genes in testis). A decrease in anogenital distance was reported but no data were presented. The *in vitro* fertility data did not show a dose-response effect. Only *ERα* expression and not *ERβ* expression were examined, despite the fact that *ERβ* is expressed in testis. Only 1 dose was used in the gene expression study. No hormonal profiles were mentioned or provided.

Utility (Adequacy) for CERHR Evaluation Process: This study has limited utility in the evaluation process since data was not included in the article for many of the endpoints that were assessed and there was a lack of a dose-response for effects on *in vitro* fertility. It suggests that further studies are needed to evaluate the potential effects of genistein exposure on the fertilization ability.

Montani et al., 2009 *(605)*, supported by European Union Grants, examined the effect of genistein administered by oral gavage to estrogen-reporter transgenic mice of different ages from adults to

suckling pups and in cultures of fetal testes at day 14.5. The doses of genistein used were representative of the intake typical of western diets (5−50 µg/kg/day), and eastern diets (50−1000 µg/kg/day) and infants fed soy-based formula (500−5000 µg/kg/day) **[purity of genistein and estradiol not stated]**. Two experiments were performed. *Experiment 1 (ER activation by estradiol and genistein in mouse testis)*: Heterozygous ERE-tK-LUC transgenic male mice **[source not stated]** were placed on estrogen-free diets for 4 days prior to treatment **[feed and bedding not described]**. A dose-response analysis was determined in testis of male mice treated orally (gavage) with 0, 5, 50, 500 and 5000 µg/kg of genistein and killed 12 hours later **[vehicle not stated, number of animals per group not stated, age of animals not stated, presumed to be adults, postmortem procedures not described]**. Lactating mothers were given a single dose of genistein by oral gavage at doses of 0, 0.5, 5, and 50 mg/kg on PND 4. ERE-tK-LUC suckling pups were killed 24 hours after the mothers' treatment and luciferase activity was measured in the testis **[vehicle not stated, number of animals per group not stated]**. *Experiment 2 (Estrogenic and Proliferative Action of Genistein ion Fetal Testis)*: Testes were dissected from 14.5 day ERE-tK-LUC fetuses and cultured in the presence of 1 µmol/L genistein, 10 nmol/L estradiol, or vehicle (ethanol) for six hours before running a luciferase assay **[number of testes per group not stated]**. *Both Experiments*: A two-way ANOVA test followed by post hoc Bonferroni analysis was used for data analysis.

Experiment 1: Genistein showed the ability to modulate the estrogen receptors (ER) in the mouse testis **[age of animals not stated, presumed to be adults]**, with a maximal level of ER action at 500 and 5000 µg/kg. The mothers' treatment resulted in greater luciferase activity in the pup testis, indicating that genistein was present in mothers' milk at concentrations sufficient to exert estrogenic actions on reproductive tissues of suckling pups. *Experiment 2*: Luciferase was induced twofold in testes exposed to genistein or estradiol, and the addition of the anti-estrogen ICI-182780 inhibited this response. Both compounds appeared to stimulate testicular cell proliferation as revealed by a twofold higher level of 3H-thymidine incorporation in cultured testes.

Authors' conclusion: Genistein affects reproductive organs of male mice at all developmental ages.

Strengths/Weaknesses: The article appears to summarize some of the data that was included in Montani *et al.*, 2008 *(600)* with the exception that data is now reported for the administration of 0.5, 5, and 50 mg/kg genistein to lactating dams on PND 4. In the previous article from the authors only a single dose level of genistein, 50 mg/kg, was evaluated. Strengths of the study include the use of oral dosing with a relevant dose range. A weakness of the study is that only a single dose of genistein was administered on PND 4. In addition, many experimental details were missing (information on the diet, age of the animals, number of animals, litter-origin, post-mortem procedures). The animal model is still experimental and not fully validated.

Utility (Adequacy) for CERHR Evaluation Process: The study is of limited utility for the present evaluation. The model may be helpful in the investigation of mechanisms of action of genistein.

3.3.1.2.2 Pre- and Postnatal - Female and male mice (oral)

Kyselova et al., 2004 ***(606)***, supported by the Czech Republic, reported a multigeneration study in CD-1 mice exposed to genistein via drinking water or diethylstilbestrol. Genistein dose levels in

drinking water were given as 0, 2.5 or 25 "µg per animal's weight per day." **[According to one of the authors, the doses should have been indicated as µg/animal. The mice weighed 20–25 g; therefore, these doses are equivalent to 0, 0.1–0.125, and 1.0–1.25 mg/kg bw/day (Buckiová D., personal communication April 27, 2005).]** The diethylstilbestrol dose level was "0.5 µg per animal's weight per day" **[0.020–0.025 mg/kg bw/day]**. The parental (F_0) mice were exposed beginning at 2 months of age, F_1 mice were exposed throughout their life, either through their dams or directly, and F_2 mice were exposed until termination at 30 days of age. Parental males were killed on PND 90 and females on PND 120. **[It is not clear whether the dose was estimated based on water consumption or some other technique was used to ensure complete intake of the daily dose. The age at mating was not given. There are PND 30 data for F_1 as well as F_2 offspring, so some F_1 animals must have been killed at this early time point. The number of animals used in each generation was not entirely clear but may have been 6/sex, at least for the F_0 matings. There is no mention of culling or weaning litters.]** Statistical analysis was performed with ANOVA and Student-Newman-Keuls test. Only the developmental endpoints (the body and organ weights on PND 30) are discussed here; reproductive endpoints are discussed in **Section 4.2**.

The high-dose genistein-treated F_0 parents showed a 5–9% decrease in body weight. No alteration of body weight on PND 30 was detected in the F_1 offspring. The F_1 male offspring showed a decrease in absolute organ weight of the testis and accessory sex glands at both genistein dose levels. Relative weights of these organs were affected in the high-dose group. F_1 female offspring had a decrease in ovarian weight on PND 30 in the low-dose group only. There appeared to be more profound suppression of testis and accessory sex gland weight in F_2 offspring, although the low-dose group did not have significant alterations in absolute or relative testis weight. High-dose F_2 females had a significant decrease in ovarian weight. Body weight was suppressed in F_2 males and females at the high dose. Diethylstilbestrol produced more pronounced effects in F_1 offspring. There were no F_2 offspring due to sterility of the F_1 animals.

Strengths/Weaknesses: A strength of this study is the long-term exposure to relatively low levels (0.1 to 1 mg/kg bw/day) of genistein, which is relevant to human exposures. Multiple generations and several endpoints were examined. The number of animals per condition (n=6) was adequate. Results were compared with diethylstilbestrol as a prototype estrogen. A weakness of the study is that administration through drinking water does not permit the calculation of an exact exposure dose. The diethylstilbestrol dose (25 µg/kg/day) was too high; effects observed at such a high dose might be secondary to alterations unrelated to reproductive tissues.

Utility (Adequacy) for CERHR Evaluation Process: This study has no utility in the evaluation process due to lack of confidence in the doses of genistein received by the animals during the study. The results show that relatively long-term exposure to genistein does not affect F_0 and F_1 mouse fertility; however, the Expert Panel had no confidence in the determination of the dose received by each animal. The effects on organ weight in F_2 animals and the note in the discussion about one F_2 male (of 6) with degenerative testes suggest the possibility of trans-generational imprinting that would deserve more study with a larger sample size and more endpoints. Similarly, the observation of some sperm damage in F_1 and recognition that it might be relevant for species with lower sperm production is useful. Data are reassuring but should be considered with caution about possible long-term effects on a small minority of individuals.

3.3.1.3 Rats: Prenatal Only

3.3.1.3.1 Prenatal - Female Rats (non-oral)

Naciff et al., 2002 ***(607)***, from the Procter and Gamble Company, examined the effects of prenatal genistein exposure via sc injection to the dam on gene expression in rat female reproductive organs. Pregnant Sprague Dawley rats were fed Purina 5K96, a casein-based soy- and alfalfa-free diet. The rats were randomly assigned to groups (≥7 rats/group) that were sc injected with genistein (~99% purity) 0 (DMSO vehicle), 0.1, 10, or 100 mg/kg bw/day on GD 11–20 (day of sperm detection=GD 0). Dams were killed on GD 20 and ovaries and uteri were removed from fetuses. In 4 litters/dose group, 1 female fetus/litter was examined for ovarian and uterine histopathology. In 5 litters/group, ovaries and uteri from ≥5 littermates were pooled for a microarray analysis of gene expression. Changes in gene expression were further quantified using RT-PCR. Data were analyzed by *t* test, ANOVA, and Jonkheere-Terpstra test. Comparisons of gene expression among estrogenic compounds were made by Wilcoxon-Mann-Whitney and Jonkheere-Terpstra tests.

Genistein treatment had no effect on maternal body weight or number of live fetuses/litter, and no gross or histopathological effects on ovary or uterus. In pooled ovary and uterus samples, expression of 227 genes was significantly altered by genistein, and the genes with the most robust response, as indicated by study authors, are listed in **Table 101**. When genistein data were pooled with data obtained from ethinyl estradiol and bisphenol A and globally analyzed, there were 66 genes that were significantly altered in the same direction by all 3 compounds; significant changes in gene expression induced by the 3 compounds are also listed in **Table 101**. The study authors concluded that gene expression in rat ovary and uterus is altered by prenatal exposure to estrogenic compounds.

Strengths/Weaknesses: Strengths include the well defined exposure time during gestation, the use of an adequate number of litters, the range of doses tested, the use of soy- and alfalfa-free diet, the comparison with ethinyl estradiol and bisphenol A, and the evaluation of histology. The confirmation of some of the array data with quantitative PCR is an additional strength. Weaknesses include the evaluation of gene expression only at the end of exposure and not at later postnatal developmental ages. A further limitation of the study is the absence of information on changes in protein expression which would further establish the importance of the documented changes in gene expression.

Utility (Adequacy) for CERHR Evaluation Process: This study analyzes the gene profile of estrogen-responsive reproductive tissues in female fetuses after gestational exposure to 3 different estrogenic compounds in an attempt to provide mechanistic clues regarding the effects of the compounds. The study could be useful in pinpointing gestational target genes that may eventually be linked to developmental defects in reproductive tissues and also unveils some common target genes between the 3 estrogenic compounds, which may be useful as sentinel genes in the evaluation of estrogen exposure. This study has limited utility in the evaluation process. Overall, the study was well designed and provides insight into potential target genes that could be modified by exposure to genistein for evaluation in future studies.

3.3.1.3.2 Prenatal - Male Rats (oral)

Thuillier et al., 2003 ***(608)***, supported by NIEHS, examined the effects of prenatal genistein exposure via oral treatment of the dam on testicular platelet-derived growth factor (*PDGF*) α- and β-receptors in fetal and neonatal male rats. According to the study authors, there is some evidence indicating that the *PDGF* pathway is involved in testicular development.

Table 101. Gene Expression Changes in Pooled Ovary and Uterus Sample in Rats Prenatally Exposed to Genistein (Naciff et al., 2002)

Parameter	*Average-Fold Change at each Genistein Dose, mg/kg bw/day*		
	0.1	*10*	*100*
Rat progesterone receptor gene, complete cds[b]	1.0	2.9	5.7
Rat intestinal calcium-binding protein (icabp) gene 2, 3, end and flank[b]	1.1	1.2	4.7
Rattus norvegicus serine threonine kinase (pim-3) mRNA, complete cds[a]	1.3	2.4	3.6
Rattus norvegicus 11-beta-hydroxysteroid dehydrogenase type 2 mRNA[b]	1.1	1.6	3.4
Rat mixed-tissue library Rattus norvegicus cDNA clone rex02348 3[a]	1.5	1.5	3.0
EST196997 Rattus norvegicus cDNA, 3 end[a]	1.1	1.2	2.7
EST 197092 Rattus norvegicus cDNA, 3 end[a]	1.3	1.5	2.7
Rat mixed-tissue library Rattus norvegicus cDNA clone rx02392 3[a]	1.5	1.5	3.0
Rattus norvegicus, GPCR-5–1 gene[a]	1.1	1.8	2.6
Rattus norvegicus mRNA for collagen alpha 1 type II, partial cds[a]	1.5	2.0	2.6
Rattus norvegicus staniocalcin (rSTC) mRNA, complete cds[a]	1.4	1.8	2.5
EST195752 Rattus norvegicus cDNA, 3 end[b]	1.3	1.7	2.4
UI-R-A0-bm-c-11–0-UI.sl Rattus novegicus cDNA[a]	1.4	1.3	2.4
Rat mixed-tissue library Rattus norvegicus cDNA clone rx01272 3[a]	1.1	1.2	2.3
Rattus norvegicus mRNA for dermo-1-protein[b]	1.1	1.4	2.1
EST188966 Rattus norvegicus cDNA, 3 end[a]	1.2	1.4	2.1
EST191592 Rattus norvegicus cDNA, 3 end[a]	1.0	1.3	2.1
EST196062 Rattus norvegicus cDNA, 3 end[a]	1.3	1.4	2.1
Rattus norvegicus mRNA for interleukin 4 receptor[b]	1.2	1.8	1.9
Rat tartrate-resistant acid phosphatase type 5 mRNA, complete cds[a]	−1.1	−1.6	−2.2
EST195631 Rattus norvegicus cDNA end[a]	−1.3	−1.5	−2.2
Rattus rattus guanine nucleotide-releasing protein (mss4) mRNA, complete cds[a]	−1.1	−1.3	−2.3
EST229949 Rattus norvegicus cDNA, 3 end[a]	−1.3	−1.8	−2.4
Rattus sp. (clone PbURF) galectin-5 mRNA, complete cds[a]	−1.3	−1.1	−2.5
Rat retinol-binding protein (RBP) partial cds[b]	−1.4	−2.0	−2.6
Rat mRNA for glycine methyltransferase (EC 2.1.1.20)[a]	−1.0	−1.2	−2.6
Rat mRNA for protocadherin 5, partial cds[a]	−1.1	−1.2	−2.7
Rattus norvericus neural cell adhesion molecule BIG-1 protein (BIG-1) mRNA[a]	−1.2	−1.3	−2.7
UI-R-E0-ct-c-11–0-UI.s1 Rattus norvegicus cDNA, 3 end[a]	−1.1	−2.0	−2.7
UI-R-E0-bs-f-12–0-UI.s1 Rattus norvegicus cDNA, 3 end[a]	−1.0	−1.2	−2.8
Rattus norvegicus mast cell carboxypeptidase A precursor (R-CPA) mRNA, partial cds[a]	−1.1	−1.9	−2.9
Rat mRNA for chromosomal protein HMG2, complete cds[a]	−1.1	−1.4	−3.1
Rattus norvegicus (clone REM2) ORF mRNA, partial cds[a]	−2.3	−2.2	−4.6
EST200668 Rattus norvegicus cDNA, 3 end (gene symbol Ttr)[a,d]	−2.9	−5.8	−5.2
EST200668 Rattus norvegicus cDNA, 3 end (gene symbol Ahsg)[a,d]	−2.2	−5.4	−5.5
Rattus norvegicus mRNA for 59-kDa bone sialic acid-containing protein, complete cds[a]	−3.1	−5.7	−5.7

Table 101 (continued)

Parameter	*Average-Fold Change at each Genistein Dose, mg/kg bw/day*		
	0.1	*10*	*100*
Rattus norvegicus mRNA for fetuin[a]	−1.7	−7.4	−6.1
Rat mRNA for serine proteinase inhibitor-like protein, partial[a]	−1.9	−3.5	−6.6
Rattus norvegicus uterus-ovary specific putative transmembrane protein (uo) mRNA[c]			1.4
Rat mRNA for vascular alpha-actin[c]			1.2
EST191592 Rattus norvegicus cDNA, 3 end. High homology to Rattus norvegicus putative G-protein coupled receptor GPCR91[c]			2.1
Rattus norvegicus (clone 59) FSH-regulated protein mRNA[c]			1.5
Rat aspartate aminotransferase mRNA, complete cds[c]			1.8
Rat phosphofructokinase C (PFK-C) mRNA, complete cds[c]			1.3
Rat very low density lipoprotein receptor (VLDLR) mRNA, complete cds[c]			1.7
Rattus rattus mRNA for glutathione-dependent dehydroascorbate reductase, complete cds[c]			1.2
Rat neural receptor protein-tyrosine kinase (trkB) mRNA, complete cds[c]			1.4
Rattus rattus RYD5 mRNA for a potenial ligand-binding protein[c]			1.4
Rat mRNA for growth potentiating factor, complete cds[c]			1.6
Rat mRNA for Na+,K+ATPase beta-3 subunit, complete cds[c]			1.5
UI-R-EO-bv-d-01–0-UI.sl Rattus norvegicus cDNA, 3 end[c]			1.5
Rattus sp. mRNA for NTAK alpha2–1p, partial cds[c]			1.6
Rat cretine kinase-B (CKB) mRNA, 3 end[c]			1.6
EST189057 Rattus norvegicus cDNA, 3 end[c]			1.4
EST198107 Rattus norvegicus cDNA, 3 end[c]			1.2
Rat brain glucose-transporter protein mRNA, complete cds[c]			1.5
Rattus vorvegicus mRNA for growth hormone receptor, 3 UTR[c]			2.2
Rat insulin-like growth factor I (IGF-I) mRNA, complete cds[c]			1.3
UI-R-E0-cb-a-03–0-UI.sl Rattus norvegicus cDNA, 3 end[c]			1.8
EST188918 Rattus norvegicus cDNA, 3 end. High homology to rat protein kinase C epsilon subspecies[c]			2.0
Rat mRNA for non-neuronal enolase (NNE) (α-α enolase, 2-phospho-D-glycerate hydrolase EC 4.2.1.11)[c]			1.3
EST196141 Rattus norvegicus cDNA, 3 end[c]			1.4
Rattus norvegicus C-CAM4 mRNA, complete cds[c]			1.4
EST196700 Rattus norvegicus cDNA, 3 end[c]			1.6
Rat DNA for prion protein[c]			1.4
Rattus norvegicus GADD45 mRNA, compelte cds[c]			2.2
EST190190 Rattus norvegicus cDNA, 3 end[c]			1.5
Rattus norvegicus serum and glucocorticoid-regulated kinase (sgk) mRNA, complete cds[c]			1.4
Rattus norvegicus nerve growth factor induced factor A mRNA, partial 3 UTR[c]			1.5
UI-R-AO-as-e-04–0-UI.sl Rattus norvegicus cDNA, 3 end[c]			1.3
Rat X-chromosome linked phosphoglycerate kinase mRNA, complete cds[c]			1.2

Table 101 (continued)

Parameter	*Average-Fold Change at each Genistein Dose, mg/kg bw/day*		
	0.1	*10*	*100*
UI-R-E0-bx-c-12–0-UI.sl Rattus norvegicus cDNA, 3 end[c]			1.4
EST213688 Rattus norvegicus cDNA, 3 end[c]			1.2
Rat protein-tyrosine-phosphatase (PTPase) mRNA, complete cds[c]			1.5
Rattus norvegicus developmentally-regulated cardiac factor (DRCF-5) mRNA, 3 end[c]			1.4
Rattus norvegicus prostacyclin synthase (ratgis) mRNA, complete cds[c]			1.2
Rat lactate dehydrogenase A mRNA, end[c]			1.2
Rattus norvegicus mRNA for protein kinase C delta-binding protein, complete cds[c]			1.2
Rat glutathione S-transferase mRNA, complete cds[c]			−1.2
Rattus norvegicus potassium channel regulatory protein KChAP mRNA, complete cds[c]			−1.2
Rat DNA polymerase alpha mRNA, 3 end[c]			−1.2
Rattus norvegicus Ssecks 322 mRNA, 3 untranslated region, partial sequence[c]			−1.2
Rattus norvegicus Drosophila polarity gene (frizzled) homologue mRNA, complete cds[c]			−1.2
Rattus norvegicus proto-oncogene tyrosine kinase receptor Ret (c-ret) mRNA, partial cds[c]			2.9
EST220459 Rattus norvegicus cDNA, 3 end[c]			−1.4
EST196721 Rattus norvegicus cDNA, 3 end[c]			−1.4
EST195725 Rattus norvegicus cDNA, 3 end[c]			−1.4
Rattus norvegicus carboxypeptidase E (CPE) gene[c]			−1.3
Rat mRNA for Distal-less 3 (Dlx-3) homeobox protein[c]			−1.3
Rattus norvegicus mRNA for precursor interleukin 18 (IL-18), complete cds[c]			−1.3
EST195719 Rattus norvegicus cDNA, 3 end[c]			−1.2
UI-R-EO-cc-c-09–0-UI.sl Rattus norvegicus cDNA, 3 end[c]			−1.2
EST197895 Rattus norvegicus cDNA, 3 end[c]			−1.8
Rattus norvegicus glutathione s-transferase M5 mRNA, complete cds[c]			−1.2
Rat mRNA for phosphodiesterase I[c]			−1.3
Rat mRNA for apolipoprotein[c]			−1.3
Rattus norvegicus C kinase substrate calmodulin-binding protein (RC3) mRNA, complete cds[c]			−1.4

[a] Statistical significance ($P<0.001$) was obtained in independent analyses of genistein.

[b] Statistical significance was obtained following independent analyses of genistein ($P<0.001$) and in analyses to determine gene expression changes occurring in same direction in pooled data from genistein, ethinyl estradiol, and bisphenol A ($P<0.0001$).

[c] Statistical significance was obtained in analyses to determine gene expression changes occurring in the same direction in pooled data from genistein, ethinyl estradiol, and bisphenol A ($P<0.0001$).

[d] It appears that the gene name for one of these compounds was mistakenly listed.

From: Naciff et al., 2002 *(607)*.

Pregnant Sprague Dawley rats were gavaged with corn oil in DMSO as the vehicle control or genistein **[purity not specified]** 0.1, 1, or 10 mg/kg bw/day on GD 14 (14 days post coitus) to PND 0 (birth). Male offspring were killed on GD 21 or PND 3, and testes were collected and fixed in formaldehyde or liquid nitrogen. Expression of *PDGFα*- and *β-receptor* RNA was measured using RT-PCR, and *in situ* and immunohistochemistry analyses were conducted to localize expression of RNA and proteins. Immunohistochemistry techniques were also used to measure expression of tyrosine-phosphorylated proteins. Data were analyzed by unpaired t test with Welch correction.

Genistein significantly increased expression of *PDGF*α- and *PDGF*β-receptor mRNA in testes of PND 3 rats at all doses **[~4–5-fold increase for α-receptor and 3–3.5-fold increase for β-receptor compared to controls]**. Diethylstilbestrol produced biphasic effects with increased expression at lower doses and decreased expression at higher doses. *In situ* analyses revealed that *PDGFα*- and *PDGFβ*-receptor mRNA were primarily localized in the interstitium of control PND 3 rats. Treatment with genistein 10 mg/kg bw/day increased expression of *PDGFα*-receptor **[~2.5-fold]** in interstitium and *PDGFβ*-receptor mRNA in interstitium **[~7.5-fold increase]** and in central and peripheral seminiferous cords **[~3–6 fold increase]**. *In situ* analysis of protein expression revealed that *PDGF* a-receptor was localized in peritubular myoid cells of PND 3 rats; treatment with genistein 10 mg/kg bw/day increased expression of *PDGFα*-receptor in Sertoli cells but not gonocytes. *PDGF*β-receptor protein was expressed at low levels in gonocytes and interstitial cells, but treatment with genistein 10 mg/kg bw/day induced strong expression in gonocytes. An examination of testes from PND 21 fetuses revealed that *PDGFα*-receptor protein was expressed in gonocytes and Sertoli cells, and no changes in expression were reported following treatment with genistein 10 mg/kg bw/day. *PDGFβ*-receptor was expressed in gonocytes of PND 21 fetuses, and expression was apparently strengthened by genistein treatment. Either no change or slight reductions in expression of tyrosine-phosphorylated protein in fetal Sertoli cells was noted following genistein exposure. Similar effects on *PDGF*α- and β-receptors were noted with other estrogenic compounds such as bisphenol A and coumestrol. The study authors concluded that the *PDGF* pathway is a target of estrogens; however, it was not known if the effects seen in this study were due to estrogenic activity.

Strengths/Weaknesses: Strengths of the study include administration of genistein by gavage, which allows the exact dose to be known, and the use of 3 dose levels. Weaknesses were that numbers of animals/group treated and examined were not specified and it was unclear if data were analyzed on a per litter basis.

Utility (Adequacy) for CERHR Evaluation Process: This study is of limited utility for the evaluation for determining developmental effects; however, the data may be useful in interpreting results from other studies.

Thuillier et al., 2009 ***(609)***, supported by the National Institutes of Health, the Royal Victoria Hospital foundation, and Le Fonds de la Rechercheen Sante du Quebec, examined the effects of fetal exposure to genistein and the xenoestrogen bisphenol A on the mitogen-activated protein kinase (MAPK) pathway and on testicular cell populations in neonatal and adult rat testes. Pregnant Sprague Dawley female rats (F_0 generation) received from CRL (Wilmington, MA) were treated by daily gavage with either corn oil (vehicle), bisphenol A (BPA), or genistein from gestation day 14 through the day of delivery. The doses for BPA **[purity not stated]** were 0.1, 10 and 200 mg/kg bw/day; the doses for genistein (purity ≥98%)

were 0.1, 1 and 10 mg/kg bw/day **[basis of dose selection not stated, number of F_0 animals per group not stated, feed and bedding not described]**. The low dose of genistein was states as being similar to the dietary level of an omnivorous American; the high dose of genistein was close to the exposure level of a soy formula-fed infant **[basis of BPA dose selection not stated]**. Male offspring were killed on PND 3, 21, or 60 **[number of F_1 animals not stated]**, serum and testes were collected, testes were either fixed in 3.5% formaldehyde or snap-frozen. Quantitative Real Time PCR (Q-PCR) Analysis of Raf1, Mek1, Erk1 and Erk2 transcripts in the whole testes were carried out. The mRNA expression of Raf1, Mek1 and Erk1 in PND 3 testes was examined by *in situ* hybridization. Testes from PND 3 and PND 60 rats were processed for immunohistochemistry. Gonocyte numbers were evaluated in PND 3 testes (3 litters from each treatment group were represented); *in vivo* gonocyte proliferation was examined by measuring the percent BrdU-positive gonocytes. Testes from PND 21 and PND 60 rats were isolated, fixed and processed for immunohistochemistry and morphometrical analysis. Levels of circulating testosterone in PND 60 rats were determined by radioimmunoassay. Fertility was examined by placing vehicle or estrogen-exposed adult male rats (n=18-25) with control females, pairs remained together for a maximum of two weeks. Females were examined daily for evidence of mating; mated females were removed from cohabitation and allowed to deliver their litters. In all experiments, the dam was used as the statistical unit; statistical analysis was performed by the two-tailed unpaired *t* test and one-way ANOVA.

Raf1, Mek1 and Erk1/2 Expression, PND 3 Testes: Q-PCR analysis of PND 3 whole testes indicated that genistein induced significant dose-dependent raises in Erk2 mRNA levels, while Erk1 mRNA levels (but not Erk2 transcripts) were significantly higher in the BPA groups compared to control. Genistein effects on Erk1 mRNA were not significant. Both BPA and genistein induced changes in Raf1 mRNA levels. Mek1 transcripts were not significantly altered by either compound. The *in situ* hybridization analysis of Raf1, Mek1 and Erk1 expression in high dose genistein and BPA PND 3 testes showed that genistein and BPA induced higher levels of Raf1 mRNA. At the protein level, both Raf1 and Erk1/2 immunoreactivity was greater in the Sertoli cell cytosol of genistein and BPA-exposed PND rats than in controls. There was no consistent change in peritubular and interstitial cell protein expression for Raf1, Mek1 or Erk1/2 in genistein or BPA-exposed testes. However genistein exposure appeared to alter patterns of phosphotyrosine protein immunoreactivity, with lower levels of tyrosine-phosphorylated proteins present at the lateral surfaces of Sertoli cells. *Raf1, Mek1 and Erk1/2 Expression, Adult Testes*: Raf1 and Mek1 did not show consistent changes between control and genistein- or BPA-exposed rats. Overall, there was no clear change in spermatogonial expression. Fetal genistein and BPA exposure did not seem to change the levels or location of the immunoreactive phospho-ERK signals. *Testicular Cell Populations*: Morphological observation of PND 3 testis sections did not reveal any significant changes in somatic cell or gonocyte populations. However, the proportion of BrdU-labeled gonocytes was lower in the testes of genistein and BPA-exposed PND 3 rats. At puberty, the only significant changes observed in testicular cell populations of genistein-exposed rats were higher numbers of spermatogonia and preleptotene/leptotene spermatocytes and greater Leydig cell numbers. There was a greater number of spermatogonia in the BPA-exposed groups. By PND 60, all adult germ cell populations had returned to normal levels, the only effect remaining was a slight, but significantly higher number of Leydig cells **[compound(s) with this effect not stated]**. *Circulating Testosterone Levels, PND 60*: Treated rats had slightly lower levels of circulating testosterone when compared to controls, but the differences were not significant. *Fertility*: All 13 control males produced litters, 3/18 low dose genistein males and 2/21 high dose genistein males did not produce litters, and 3/25 high dose BPA males did not produce litters; these differences were not statistically significant.

Authors' conclusion: The data suggest that fetal exposure to genistein and BPA exerts transient effects in rat testes and the changes observed at PND 3 did not correlate with relevant changes in germ cell populations, Leydig cell functions, or fertility in the adult.

Strengths/Weaknesses: Strengths of the study is the use of the oral route of exposure, assessment of a dose-response for some of the parameters, and the attempt to assess both mRNA and protein expression for the selected endpoints. The images showing the results of the *in situ* hybridization and part of immunohistochemistry appear technically poor, which makes interpretation difficult. Formalin fixation of testes for morphological and morphometric analysis is not adequate, and guidelines for reproductive toxicology advise against it. Bouin's fluid should have been used to fix the testes for the various evaluations that were performed. It is possible that the crosslinking due to formalin fixation impacted the penetration of the probes used in the *in situ* hybridization and the antibodies used in the immunohistochemistry.

Utility (Adequacy) for CERHR Evaluation Process: The study is of limited utility because of methodological weaknesses.

3.3.1.3.3 Prenatal - Male Rats (non-oral)

Naciff et al., 2005 ***(610)***, from the Procter and Gamble Company, examined the effect of prenatal genistein treatment via sc injection of the dam on male reproductive organ histology and gene expression. Pregnant Sprague Dawley rats were fed Purina 5K96, a casein-based soy- and alfalfa-free diet. The rats were randomly assigned to groups (≥8 rats/group) that were sc injected with genistein **[purity not reported]** 0 (DMSO vehicle), 0.001, 0.01, 0.1, 10, or 100 mg/kg bw/day on GD 11–20 (day of sperm detection=GD 0). Dams were killed on GD 20 and testes and epididymides were removed from fetuses. In 4 litters/dose group, 1 male fetus/litter was examined for testicular histopathology. In 5 litters/group, testis and epididymis from 5 littermates were pooled for a microarray analysis of gene expression. Changes in gene expression were further quantitated using RT-PCR. Data were analyzed by *t* test, ANOVA, and Jonkheere-Terpstra test. Comparisons of gene expression among estrogenic compounds were analyzed by Wilcoxon-Mann-Whitney and Jonkheere-Terpstra tests.

Genistein treatment had no effect on maternal body weight or number of live fetuses/litter, and no gross or histopathological effects on testis or epididymis. In pooled testis and epididymis samples from the high-dose genistein group, expression of 23 genes was significantly altered in a dose-related manner, and those genes are listed in **Table 102**. When genistein data were pooled with data obtained from ethinyl estradiol and bisphenol A and globally analyzed, there were 50 genes that were significantly altered in the same direction by all 3 compounds; significant changes in gene expression induced by the 3 compounds are also listed in **Table 102**. The study authors concluded that transplacental exposure to high doses of genistein alters the expression of certain genes in the testis and epididymis of fetal rats without causing malformations in those organs. The study authors noted that the dose response to genistein was monotonic with no evidence of robust quantifiable responses at low doses.

Strengths/Weaknesses: This study is similar to the previous study by Naciff *et al.*, 2002 *(607)* and has similar strengths and weaknesses. The lack of a dose-response and the small magnitude of the observed changes in gene expression plus the lack of histomorphological changes in the fetal testes and epididymides makes it difficult to assess the biological relevance of the changes in gene expression.

Table 102. Gene Expression Changes in Pooled Testis and Epididymis following Prenatal Exposure to Genistein (Naciff et al., 2005)

Gene	*Average-fold Change at each Genistein Dose, mg/kg bw/day*				
	0.001	*0.1*	*1.0*	*10.0*	*100*
Hydroxyacid oxidase 3 (medium chain)[b]	−1.1	1.1	1.1	1.8	3.5
Progesterone receptor[b]	1.2	1.4	1.1	2.1	2.9
Progesterone receptor steroid-binding domain[b]	1.1	−1.1	1.0	1.5	2.1
ESTs (accession no. AA858607)[b]	1.2	1.2	1.3	2.1	1.7
MCT1 monocarboxylate transporter[b]	1.0	1.1	1.2	1.6	1.7
Sulfonylurea receptor 2[b]	1.0	1.1	1.1	1.2	1.7
Small proline-rich protein gene[a]	−1.0	1.1	1.3	1.2	1.6
Solute carrier 16 (monocarboxylic acid transporter)[a]	1.0	1.1	1.1	1.4	1.5
Gap junction protein, alpha 1, 43 KD (connexin 43)[a]	−1.1	−1.1	1.1	1.4	1.5
ESTs, Weakly similar to T43458 hypothetical protein DKFZp434F0621.1[a]	1.1	−1.0	−1.0	1.3	1.5
Mitochondrial 3-hydroxy-3-methylglutaryl-CoA synthase[b]	−1.1	1.0	1.0	1.1	1.5
ESTs (accession no. AA866383)[a]	1.1	1.1	1.1	1.3	1.4
ESTs (accession no. AA893596)[a]	1.0	−1.1	−1.0	1.2	1.4
ESTs, similar to potassium channel, subfamily K, member 5 (Mus musculus)[b]	−1.2	−1.1	−1.1	1.1	1.4
3-hydroxy-3-methylglutaryl-Coenzyme A synthase 2[a]	−1.0	1.0	−1.0	1.1	1.4
Solute carrier family 18 (vesicular monoamine), member[a]	−1.1	1.0	−1.0	1.1	1.4
Zinc finger protein 354A[a]	−1.0	1.0	−1.0	−1.3	−1.4
Luteinizing hormone/choriogonadotropin receptor[a]	1.0	1.1	1.1	−1.1	−1.5
Phospholipase A2, group IB[a]	−1.1	−1.1	−1.2	−1.3	−1.6
Carboxypeptidase A1[b]	1.1	1.1	1.1	−1.1	−1.6
Natriuretic peptide precursor C[b]	1.1	−1.0	1.1	−1.8	−1.9
Cytochrome P450, subfamily XVII[b]	1.1	−1.0	1.1	−1.2	−2.5
Steroidogenic acute regulatory protein[b]	1.1	1.1	1.0	−1.3	−3.0
Intestinal calcium-binding protein, calbindin 3[c]			1.1	1.0	4.3
Endothelial differentiation, lysophosphatidic acid G-protein-coupled[c]			1.1	1.0	1.2
Guanylate kinase associated protein[c]			1.1	1.2	1.7
Very low density lipoprotein receptor[c]			1.1	1.0	1.2
Vascular endothelial growth factor[c]			1.1	1.0	1.2
Interleukin-4 receptor[c]			1.1	1.3	1.3
Rhesus blood group-associated A glycoprotein[c]			1.0	1.0	1.3
3-hydroxy-3-methylglutaryl CoA synthase[c]			−1.0	1.1	1.4
EGL nine homolog 3 (C. elegans)[c]			1.2	1.2	1.3
Silencer factor B[c]			1.1	1.5	1.3
ATP-binding cassette, sub-family C (CFTR/MRP), member[c]			−1.1	−1.1	1.4
ESTs, high homology to fos-like antigen 2[c]			−1.1	1.1	1.2
Momonocarboxylic acid transporter, member 1[c]			1.2	1.6	1.7

Table 102. (continued)

Gene	*Average-fold Change at each Genistein Dose, mg/kg bw/day*				
	0.001	*0.1*	*1.0*	*10.0*	*100*
ESTs, high homology to N-myc downstream-regulated gene 2[c]			−1.0	1.1	1.3
Lectin, galactose binding, soluble 9 (Galectin-9)[c]			1.1	1.1	1.3
N-myc[c]			−1.1	−1.0	1.3
ESTs, high homology to solute carrier organic anion transporter family, member 2a1[c]			1.1	1.1	1.3
CD36 antigen (collagen type I receptor, thrombospondin[c]			1.3	1.2	1.6
ESTs, high homology to phosphatidic acid phosphatase type 2c[c]			1.0	1.1	1.4
EGL nine homolog 3 (C. elegans)[c]			1.1	1.0	1.2
Similar to carboxypeptidase-like protein ACLP[c]			−1.0	1.1	1.2
ATPase, Na+K+ transporting, beta polypeptide 3[c]			−1.0	1.0	1.1
ESTs (accession no. AA894233)[c]			−1.1	−1.2	−1.3
Fatty acid Coenzyme A ligase, long chain 3[c]			−1.1	−1.1	−1.1
Isocitrate dehydrogenase 1, soluble[c]			1.0	−1.1	−1.1
Fatty acid-Coenzyme A ligase, long chain 4[c]			1.0	1.0	−1.2
RANP-1, sterol-C4-methyl oxidase-like[c]			−1.0	−1.1	−1.3
3-hydroxy-3-methylglutaryl-Coenzyme A synthase 1[c]			−1.0	−1.1	−1.2
7-dehydrocholesterol reductase[c]			1.2	−1.0	−1.1

[a] Statistical significance ($P \leq 0.001$) was obtained at the highest dose in independent analyses of genistein.

[b] Statistical significance was obtained following independent analyses ($P \leq 0.001$) of genistein and in analyses to determine gene expression changes occurring in same direction in pooled data from genistein, ethinyl estradiol, and bisphenol A ($P \leq 0.001$) **[text states $P \leq 0.0001$]**.

[c] Statistical significance was obtained in analyses to determine gene expression changes occurring in the same direction in pooled data from genistein, ethinyl estradiol, and bisphenol A ($P \leq 0.001$) **[text states $P \leq 0.0001$]**.

From Naciff et al., 2005 *(610).*

Utility (Adequacy) for CERHR Evaluation Process: This study has limited utility in the evaluation process due to the use of the sc injection as the route of exposure. The study may be useful for the exploration of possible mechanisms of action, as discussed above for Naciff *et al.*, 2002 *(607)*.

3.3.1.3.4 Prenatal - Female and Male Rats (oral)

Kang et al., 2002 ***(611)***, supported by the Brain Korea 21 project and the Korean FDA, examined the effects of maternal genistein via gavage on development of reproductive organs in offspring. Pregnant Sprague Dawley rats were fed AIN-76A, a casein-based soy-free diet. The rats were randomly assigned to groups of 9–12 and were gavaged with genistein (> 98% purity) in corn oil at 0, 0.4, or 4 mg/kg bw/day from GD 6 (day following mating=GD 1) to PND 20 (day of parturition=PND 1); the dams were not dosed on PND 1–2. Genistein doses were based on intake in Asian populations. A positive control group was treated with 10 µg/kg bw/day 17β-estradiol. Upon weaning of litters, dams were killed and examined for implantation sites and organ weights. At birth, pups were sexed by measuring anogenital distance, weighed, and examined for toxicity, mortality, and gross abnormalities. During the postnatal period, pups were weighed and monitored for eye and vaginal opening. Offspring (n=5–7/group/sex/time period) were killed on PND 21, 33, 49, 70, or 100. Body and reproductive organ weights (testis, seminal vesicle,

prostate, uterus, and ovary) were measured; reproductive organs were examined histologically on PND 100. Testes were fixed in Bouin fluid, and all other tissues were fixed in 10% neutral buffered formalin. Sperm count and motility were assessed. Differential follicle counts were conducted on ovaries. Data were analyzed by 2-way ANOVA. **[It was not stated if the litter was considered in statistical analyses.]**

No effects of genistein treatment on dam body or organ weights, number of implantation sites, live pups, pups survival to weaning, sex ratio, anogenital distance, eye opening, or vaginal opening were detected. There were no observed effects of genistein exposure on postnatal weight gain in male or female offspring. Relative (to brain weight) organ weight effects included increased testis and seminal vesicle weight in the low-dose group on PND 33 and increased prostate weight in the high-dose group on PND 70. **[Absolute organ weights were not reported.]** Organ weight changes were transient, and no histopathologic effects were observed in testis, seminal vesicle, or prostate **[data not shown]**. On PND 100, genistein had no observed effect on sperm count or motility or on the cell types at stage VII of the spermatogenic cycle. Relative uterine weight was significantly increased in the low-dose genistein group on PND 33. **[Absolute organ weights were not reported.]** Organ weight changes were transient, and no abnormal histopathologic findings were observed in ovary or uterus **[data not shown]**. On PND 100, numbers of primordial follicles were slightly reduced in the high-dose group, but no significant alterations in follicle development were detected. Significant effects observed with 17β-estradiol included reduced relative seminal vesicle weight from PND 21–70, decreased numbers of elongated spermatids on PND 100, decreased relative uterus and ovary weights on PND 21, and increased relative ovary weight on PND 33. It does not appear that histopathologic examination was conducted in rats from the 17β-estradiol group. The study authors concluded that gestational and lactational exposure of rats to genistein at levels within the range of human intake appears to have no adverse effects on reproductive organs.

Strengths/Weaknesses: Strengths of this study include use of soy-free chow, random assignment of animals to treatment groups (9–12/group), and use of 17β-estradiol as a positive control. Because genistein was administered by gavage, the exact dose was known. Weaknesses of the study included use of only 2 genistein dose levels (0.4, 4 mg/kg bw), no treatment during parturition, no indication if the litter was used as the experimental unit, and no assessment of reproductive capability.

Utility (Adequacy) for CERHR Evaluation Process: This study has no utility in the evaluation process.

McClain et al., 2007 ***(612)*** examined the effects of synthetic genistein (DSM Nutritional Products)—exposure in rats in two prenatal oral administration studies **[DSM Nutritional also appears also to be the source of funding for this research since 4 of 5 authors are affiliated with the company**]. The first study was a pilot oral gavage study in female Wistar (RORO) rats (10/group) at dose levels of 0 (control), 20, 150, and 1000 mg/kg bw/day administered from GD 6-20. Genistein suspension was prepared fresh daily and was suspended in a vehicle of 5g carboxymethylcellulose, 5ml benzyl alcohol, 9g NaCl, 4ml Tween 80 in 1000ml distilled water. **[Age at study initiation was not provided.]** The range of body weights at the start of study was 183-208 g. Female body weights were taken on GD 0 and GD 6-21 and on lactation day (LD)1, 4 and 6. Food consumption measurements for dams were made on GD 0, 7, 14 and 21 and on 1 and 6. Litter size determinations were made daily during LD 1-7. Gross examination of the dams was performed on LD 7. The offspring were sacrificed on PND 7 and given external and visceral examinations, and processed for skeletal examination. Sex determination was done on PND 1 and 7. Data were analyzed using Chi-square, Fisher's Exact Test,

ANOVA, Dunnett's Test, Kruskal-Wallis test and Mann-Whitney-Wilcoxon test. The data are generally expressed as the median. This study was not a GLP compliant study. There were no maternal deaths and no adverse clinical signs. There was no significant effect on dam body weight up to 150 mg/kg bw/day. At 1000 mg/kg bw/day, dam body weights were slightly lower as compared to controls during the first week of treatment and remained reduced throughout the lactation period. Maternal food consumption was slightly reduced at 1000 mg/kg bw/day during treatment. There was no effect of genistein on the fertility index, gestation index, number of females surviving delivery, duration of gestation, number of delivered pups, the number of implantation sites, or the number of resorbed implants.

At 20 and 1000 mg/kg bw/day pup mortality during lactation was significantly higher as compared to controls; however, pup mortality was significantly lower at 150 mg/kg bw/day as compared to the controls. Pup mortality was 11, 25, 2 and 26% across the dose groups respectively. One female at the low dose and two females at the high dose had complete litter loss over LD 1-7. Poor general condition was observed in 1 pup, 2 pups, or all pups in the litters of three females at 1000 mg/kg bw/day. Median pup body weights tended to be lower at 20 and 1000 mg/kg bw/day but not significantly. A total of 80, 91, 93, and 98 pups were evaluated for external anomalies in the 0, 20, 150, and 1000 mg/kg bw/day dose treatment groups. No external malformations were observed in any of the groups. Visceral evaluation (71, 68, 91, and 72 pups examined across dose groups, respectively) showed an increase in the retardation finding of "thymus remnant" with no pups affected at 0 and 20 mg/kg bw/day and 2 and 5 pups from one litter at 150 and 1000 mg/kg bw/day, respectively. The blood vessel variation "artery origin variant" increased in a dose-dependent manner, with no pups in the control group affected and 1.5, 5.5, and 5.6% of the pups affected at 20, 150, and 1000 mg/kg bw/day, respectively. Skeletal evaluation (70 and 73 pups examined in control and 1000 mg/kg bw/day, respectively) revealed a slightly higher incidence of abnormalities of fused and/or misshaped sternal elements and "bilateral thoracic extra ribs" at 1000 mg/kg bw/day as compared to the controls. No skeletal examinations were done in the 20 and 150 mg/kg bw/day groups.

This second study was a GLP prenatal developmental study conducted to conform to OECD draft guideline 414, June 2000. In this study, genistein was administered in the diet to female Wistar: WIST Hanlbm: (SPF) rats (30/group) at dose levels of 0 (control), 5, 50, 100, and 500 mg/kg bw/day administered from GD 5-21. **[Age of the animals at study initiation was not provided—just the same weight range at initiation as given for the pilot study**.] The day of mating was designated GD 0. The genistein used in this study had a purity of 98.3%. Genistein was mixed with genistein free microgranulated feed at concentrations calculated to achieve target dose levels based on the most recent body weight and food consumption data. Mated rats were assigned to dose groups using a computer-generated random algorithm. Plasma samples were collected from 5 females per group on GD 21 and were assayed by LC/MS for free genistein and genistein conjugates after enzymatic cleavage to provide data for both the levels of free and total genistein. Females were checked for mortality, morbidity, signs of abortion or premature delivery, and adverse clinical signs at least twice daily. Body weights were recorded daily from GD 0-21. Food consumption was recorded for the following intervals: GD 0-3, 3-5, 5-9, 9-12, 12-15, 15-18, and 18-21. The intake of genistein ranged from 96 to 103% of the target dose. On GD 21, females were sacrificed and gross examination was performed. Gravid uterine weights were collected. Apparently non-gravid uteri were stained to visualize implantation sites. Implant classification was done on each uterus. The fetuses were sexed, weighed individually, examined externally, and anogenital distance (AGD) measured. Half the fetuses were processed for

Wilson's slicing technique for visceral examination. The remaining half was processed using a double staining technique for stain of bone and cartilage. Structural alterations were defined according to three categories—abnormality, variation and skeletal variant. The following parameters were analyzed: body weights, food consumption, uterus weights, anogenital distances, reproduction, and skeletal examination data. Means and standard deviation were calculated. The following statistical methods were used: Dunnett's many-one t test, Steel test, and Fisher's Exact test. Ratios of AGD to the cube root of body weight were calculated to normalize for different fetal weights.

At the end of the treatment period, mean female body weights were lower (statistically significantly) in the high dose group of 500 mg/kg bw/day —a decrease of 21.3% as compared to controls. Adjusted body weight gain was also lower (although not significant) on GD 21 in high dose females. Mean food consumption during the treatment period was markedly lower (not significant) in the high dose group—minus 17.8% vs controls. At 50 and 100 mg/kg bw/day **[The text of the paper stated 150 mg/kg bw/day instead of the correct dose level of 100 mg/kg bw/day shown in Table 6.]** food consumption was similar to controls. Food consumption was slightly lower in the 5 mg/kg bw/day; however, this was considered incidental due to lack of dose response. Plasma data analysis showed that animals were highly exposed to genistein. At 500 mg/kg bw/day postimplantation loss was markedly higher (statistically significant)—36% vs 5.4% in controls—because of a greater number of embryonic resorptions. 13 high dose females had total resorptions. There was a correspondingly lower (statistically significant) mean number of fetuses per female in the high dose group, 7.3 vs 11.8 in controls. Fetal body weights calculated on a litter basis were lower (statistically significant) in fetuses from high dose females (4.2 g vs 4.9 g in controls.) All other dose groups were comparable to controls. In the absence of a dose relationship, differences in AGD were considered incidental by the authors and not related to treatment for both male and female fetuses. There were no external, visceral or skeletal abnormalities considered related to treatment with genistein. The high dose level had a higher incidence of skeletal ossification observations that corresponded with lower fetal body weights.

The authors' conclusions based on these 2 studies are that genistein has no teratogenic potential *in vivo* at very high doses of up to 1000 mg/kg bw/day by oral gavage or up to 500 mg/kg bw/day by dietary administration even though these doses were maternally toxic and fetal-toxic. On the basis of the above diet study, the NOAEL for maternal toxicity and adverse effects on embryonic and fetal development was considered to be 100 mg/kg bw/day.

A third study reported in this paper was an *in vitro* rat whole embryo culture assay (WEC), which was used as a preliminary screen for fetotoxic and teratogenic potential. The concentration range used was 1 to 100 µg/ml. The genistein had a purity of 98.3% for this assay. Thirty implantations were removed from mated rats on about GD 9.5. Three flasks each with 2 embryos were cultured in the absence of genistein (control) and for the remaining flasks (8 embryos per dose) in the presence of genistein dissolved/suspended in gelatin at various concentrations. At the end of a 48 hour culture period, the embryos were assessed for anomalies, growth, and degree of differentiation using a modified morphological scoring system of Brown and Fabro (Brown, N.A., Fabro, S., 1981. Quantitation of rat embryonic development *in vitro*: A morphological scoring system. Teratology 24, 65–78. 1981).

At genistein concentrations of 0, 1, 3, 10, 30, and 100 µg/ml (conducted in 2 assays), anomalies considered to be treatment-related were observed at concentrations of ≥10 µg/ml. At 100 µg/ml,

all embryos were malformed. There were no embryonic changes at 1 or 3 µg/ml considered to be anomalies. There were no clear signs of general toxicity to the embryos at 10 and 30 µg/ml; embryonic growth and differentiation were slightly impaired at these two concentrations. The author's conclusions were since anomalies were observed (10 or 30µg/ml) in the absence of general embryo or yolk sac toxicity, genistein is considered to exhibit a teratogenic potential *in vitro* in the WEC assay at concentrations of ≥10 µg/ml.

The authors' concluded that genistein displayed teratogenic potential at high concentrations *in vitro*, but these effects were not predictive of the findings from the *in vivo* studies that indicated a lack of teratogenic potential even at a very high dose of 1000 mg/kg bw/day. The authors suggested that the lack of concordance between the *in vitro* and *in vivo* studies may reflect differences in the conjugation and metabolism of genistein between the pregnant dams and the embryos used in the *in vitro* model. In addition, the concentrations used *in vitro* that were associated with teratogenesis were high when compared to concentrations of free genistein that have been measured in fetuses exposed via the dam.

Strengths/Weaknesses: Strengths of the *in vivo* studies are the use of oral routes of exposure, the assessment of a dose-response, use of 30 animals/group in the GLP study, appropriate period of exposure to assess teratogenic potential, appropriate evaluations, and measurement of plasma genistein levels in the dams on GD 21 A weakness of the study is lack of presentation of the litter mean data in the results tables.

Utility (adequacy) for CERHR Evaluation Process: This study has limited utility for the evaluation process. The lack of teratogenicity in fetuses of dams treated with high levels of genistein is useful information. The *in vitro* whole embryo culture study has no utility in the evaluation process. A limitation of the study for the evaluation of soy infant formula is that animals were treated during gestation only.

Möller et al., 2009 ***(613)***, supported by the Deutsche Forschungsgemeinschaft, examined the effects of lifelong exposure to three different diets (phytoestrogen-free, high in isoflavones, and genistein-rich) on uterine weight and gene expression at two distinct time points (juvenile and adult) in female Wistar rats. The F_0 generation (male and female) was obtained from Janvier (Le Genest St Isle, France) when they were 10 weeks old and randomly split into three feeding groups: 1) a phytoestrogen-free diet (Control, Ssniff SM R/M-H, 10 mm), 2) a diet high in isoflavones (ISO-high, produced from dehulled soybean meal containing 232 µg daidzein and 240 µg genistein/gram of diet, Harlan Teklad 8604 rodent diet), and 3) a genistein-rich diet (700 µg/g genistein added to the phytoestrogen-free diet) **[purity of the genistein and daidzein not stated]**. The phytoestrogen-free diet served as the control and mimicked the phytoestrogen (PE) content of the typical Western diet; the ISO-high diet simulated the PE exposure of a typical Asian diet; the genistein-rich diet represented the Western diet supplemented with genistein. The pregnant females remained on their assigned diets throughout gestation and lactation **[duration of exposure prior to mating not stated]**. On PND 21 **[bedding not described]**, six female pups per group were killed, plasma was collected, and the uterus was weighed and snap-frozen for RNA and protein preparation; an additional six female pups per group were weaned onto the maternal diet and remained on their assigned diets through adulthood. On PND 80, these females were ovariectomized (OVX) to reduce the endogenous hormone background, after a 17-day recovery, the animals were killed, plasma was collected, and the uterus was weighed and snap-frozen. The total RNA was extracted from the uteri (juvenile and adult); RNA was pooled within treatment groups and the DNA contamination was enzymatically eliminated by digestion−success of

the DNA elimination was verified by PCR. cDNA was amplified by quantitative real-time PCR (qPCR), the expression of all genes was normalized against the housekeeping gene *cytochrome-c-oxidase subunit 1A*, and all gene expression profiles were normalized to the control (PE-free diet) group. Finally, a Western blot analysis was conducted. The expression levels were analyzed for the following: estrogen receptors ERα and ERβ, progesterone receptor (PR), clusterin (*Clu*) and complement C3, the proliferating cell nuclear antigen (*PCNA*), the antigen identified by monoclonal antibody Ki67 (*Ki76*), and insulin-like growth factor 1 (*IGF-1*) and its receptor (*IGF-1R*). Statistical Analysis of the uterine weight data included ANOVA followed by Bonferroni post hoc test. The standard Student's *t* test was used to detect statistically significant differences of gene expression profiles.

Dietary intake and Plasma Levels: The control animals (PE-free diet) had negligible exposure to phytoestrogens (PEs); juvenile females ingested 49 and 73 mg isoflavones/kg/day in the ISO-high and genistein-rich groups, respectively. During adulthood, PE levels reached 63 and 93 mg/kg bw/day in the ISO-high and genistein-rich groups, respectively. PEs and metabolite levels in the blood plasma of the animals fed the PE-free control diet group were close to the detection limit. Animals in the ISO-high diet group showed high PE levels (468 ng genistein, 338 ng daidzein, 821 ng equol/ml plasma), while the genistein-rich diet group had PE levels of 1038 ng genistein, <6 ng daidzein, and <3 ng equol/ml plasma. *Uterine Weights*: In juvenile animals, there was no significant difference in mean uterine weights between the ISO-high diet group and the PE-free control diet group; however, the mean uterine weight of the genistein-rich diet group was significantly higher than the PE-free control diet group. In the adult OVX animals, both the ISO-high diet and the genistein-rich diet groups had significantly heavier uterine weights (673 mg/kg and 623 mg/kg, respectively) when compared to the PE-free diet group (estimated from graph to be approximately 450 mg/kg). *Gene and Protein Expression*: The mRNA expression of both estrogen receptor subtypes (ERα/ERβ) was not affected by the ISO-high diet in the juvenile or the adult uterus; however, the juvenile animals of the genistein-rich diet showed a highly significant down regulation (0.20 fold for ERα, 0.29 fold for ERβ). For ERα (but not ERβ), this lower expression was also detected in the adult animals. In juvenile animals, the ISO-high diet led to an up-regulation of PR expression (2.03 fold), whereas a significant down-regulation (0.27 fold) was noted in the genistein-rich diet group. In adult animals, the PR mRNA levels were converse−there was a significant down-regulation (0.48 fold) in animals receiving the ISO-high diet whereas the genistein-rich diet had no effect. Neither the ISO-high nor the genistein-rich diet significantly affected the regulation of *Clu* at any observed time point. A strong up-regulation (44.05 fold) of C3 transcription was seen in the juvenile animals after feeding the genistein-rich diet, while a significant down-regulation (0.38 fold) was seen in the adult animals from this group. In the ISO-high diet group, a down-regulation (0.75 fold) was seen in the pre-puberty development stage that continued into adulthood (0.62 fold). The two proliferation markers *PCNA* and *Ki67* responded in a similar expression pattern to the PE-containing diets; the more sensitive time point was the juvenile stage. Both proliferation markers were down-regulated by both PE diets, with *Ki76* being the most sensitive one (*PCNA*−0.47 fold for ISO-high diet, 0.53 fold for genistein-rich diet; *Ki67*−0.45 fold for ISO-high diet, 0.21 fold for genistein-rich diet). In adult animals, a weak up-regulation of *PCNA* was seen in the ISO-high diet group (1.35 fold) and in the genistein-rich diet group (1.14 fold). Up-regulation of *Ki67* was also seen (1.37 fold for ISO-high diet, 1.44 fold for genistein-rich diet). Similar levels of the PCNA protein were seen in all dietary groups, although a slight induction (1.47 fold) was seen in the ISO-high diet group. The genistein-rich diet led to a significant down-regulation of *IGF-1* (0.29 fold) and *IGF-1R* (0.35 fold) mRNA levels in juvenile rats, but these effects were not

observed in adults OVX animals. No effect of the ISO-high diet was seen on the regulation of *IGF-1* gene expression at either age; *IGF 1R* mRNA levels were unaffected by the ISO-high diet in juveniles, but were significantly down-regulated in the adult.

Authors' conclusion: Both the time point on which phytoestrogen exposure starts together with the composition of the ingested phytoestrogen-containing diet are of great importance for the biological response of the offspring.

Strengths/Weaknesses: Strengths include oral, continuous exposure to doses of isoflavones and genistein that are relevant to human exposures. Circulating levels of metabolites were measured, and end points were evaluated in animals that had been exposed throughout adolescent and adult periods of time. Weaknesses are that there was no dose response, and details of experimental design were somewhat vague. It was difficult to appreciate animal numbers for each group and whether there had been a control for litter effect. Also, the relevance of all of the uterine genes investigated was not clear. It is difficult to assess the value of the findings for humans.

Utility (adequacy) for CERHR Evaluation Process: This study is of limited utility for the evaluation process because some aspects of the experimental design were worthwhile, but overall relevance to human exposures could not be appreciated.

3.3.1.4 Rats: Postnatal Only

3.3.1.4.1 Postnatal - Female Rats (non-oral)

Cotroneo et al., 2001 ***(215)***, supported by NIH, evaluated the effects of sc injection of genistein towards the end of the lactation period on uterine weight in the Sprague Dawley rat. Female rat pups were injected sc with estradiol benzoate 0.5 mg/kg bw (positive control), genistein **[purity not specified]** 500 mg/kg bw, or vehicle (DMSO). Pups were treated on PND 16, 18, and 20. During their gestation, the dams had been given a phytoestrogen-free diet (AIN-76A). On PND 21, 1 group of pups was killed 18–20 hours after the last injection. Other pups were weaned to the phytoestrogen-free diet and were killed on PND 50 or 100. A separate group of 16-day-old pups was ovariectomized and treated with sc injections of estradiol benzoate, genistein, or vehicle at 16, 18, and 20 days of age, as above. On PND 21, these pups were killed 18–20 hours after the last injection. **[There was no information on how many litters gave rise to these pups or whether littermates were treated together or were randomized to treatment groups.]** An additional group of rats was exposed to genistein in the diet. Because the dietary study focused primarily on uterine weight, this endpoint is included in Chapter 2 which summarizes estrogenicity studies. Uteri were weighed, and whole uterine extracts were analyzed by Western blot for ERα, progesterone receptor, and androgen receptor protein. Immunohistochemistry for ERα localization was performed on uterine sections. Scoring of sections was based on assigning 0, + (weak), ++ (moderate), +++ (strong), or ++++ (intense) to each of three uterine structures (epithelium, muscle, stroma) and averaging the individual ranks. RT-PCR was used to quantitate uterine mRNA for ERα, ERβ, progesterone receptor, androgen receptor, and β-actin (which was used to normalize the receptor measurements). Total and unconjugated genistein was measured in serum **[method of collection not given]** by HPLC-MS with a detection level of 10 pM **[2.7 ng/L]**. RIA kits were used to determine serum 17β-estradiol, progesterone, and testosterone. Statistical analysis was performed using ANOVA **[post-hoc test not indicated]**.

Table 103. Response of Intact and Ovariectomized Female Rats to Genistein or Estradiol Benzoate Assessed on PND 21 (Cotroneo et al., 2001)

Parameter	Intact		Ovariectomized	
	Genistein	Estradiol Benzoate	Genistein	Estradiol Benzoate
Relative uterine weight	↑2.3-fold	↑2.2-fold	↑2.4-fold	↑2.6-fold
Serum 17β-estradiol	↑1.6-fold	↑1.8-fold	Not evaluated	
Serum progesterone	↓62%	↓52%		
Serum testosterone	↔	↔		
ERα protein[a]	↓76%	↓62%	↓88%	↓89%
Progesterone receptor protein[a]				
Isoform A	↑1.7-fold	↑1.7-fold	↑1.4-fold	↑2.1-fold
Isoform B	↑1.5-fold	↑1.8-fold	↑1.5-fold	↑1.5-fold
Androgen receptor protein[a]	↓22%	↓17%	↓22%	↓30%

Rat pups were injected SC with vehicle, genistein (500 mg/kg bw), or estradiol benzoate (0.5 mg/kg bw) on PND 16, 18, and 20 and killed on PND 21; n=8/group. Ovariectomy was performed on PND 16.

↑,↓,↔ Significant increase, decrease or no difference compared to vehicle control.

[a]Estimated from graph.

From Cotroneo et al., 2001 *(215)*.

The responses of intact and ovariectomized rats assessed on PND 21 are summarized in **Table 103**. Hypertrophy of the luminal and glandular epithelium of the uterus was reported in animals treated with either genistein or estradiol benzoate. Immunohistochemical staining intensity for ERα was less intense in uteri from animals treated with genistein or estradiol benzoate compared to control. Uterine mRNA for ERα was decreased 37% in genistein-treated rats compared to controls **[estimated from graph]**; an apparent reduction in estradiol benzoate-treated rats of similar magnitude was not statistically significant. No treatment effects on mRNA for *ERβ*, progesterone receptor, or androgen receptor were detected. ERα protein was decreased 66% from the control value on PND 50 (30 days after the last treatment) in genistein-exposed rats but recovered to control levels by PND 100. Estradiol benzoate treatment had no observed effect on ERα protein on PND 50 or 100. There were no detected effects on progesterone or androgen receptor protein on PND 50 or 100 after treatment with either genistein or estradiol benzoate. Although serum 17β-estradiol levels were increased and progesterone levels were decreased by genistein and estradiol benzoate treatment on PND 21, neither treatment was shown to alter serum 17β-estradiol or progesterone on PND 50; serum hormone assays were not performed at PND 100. Total serum genistein concentrations in intact rats after genistein treatment on PND 16, 18, and 20 were as follows: PND 21 (n=6) 5558 ± 1434 nM **[1502 ± 388 µg/L aglycone equivalent]**; PND 50 (n=7) 39 ± 12 nM **[11 ± 3 µg/L aglycone equivalent]**; and PND 100 (n=9) 13 ± 1 nM **[4 ± 0.3 µg/L aglycone equivalent; error not given, but SEM was used elsewhere in this manuscript for reporting data].** Free genistein was reported as follows: PND 21: 1956 ± 114 nM **[529 ± 31 µg/L]**; PND 50: 16 ± 6 nM **[4.3 ± 1.6 µg/L]**; and PND 100: 6 ± 1 nM **[1.6 ± 0.3 mg/L]**.

[The Expert Panel noted that effects seen with a high dose (500 mg/kg bw) of injected genistein mimicked those seen with injected 17β-estradiol (e.g., increased uterus:body weight ratio, increased serum 17β-estradiol, decreased serum progesterone, decreased uterine ERα and androgen

receptor, increased uterine progesterone receptor A and B, decreased uterine ERα mRNA and immunohistochemical labeling, and hypertrophy of uterine luminal and glandular epithelia). Similar results were seen in ovariectomized rats treated with genistein via the same dosing paradigm and sacrificed on PND 21 (e.g., increased uterus:body weight ratio, decreased serum progesterone, decreased uterine ERα and androgen receptor, increased uterine progesterone receptor A and B). However, as noted in Chapter 2 at 250 mg genistein/kg diet, uterus:body weight ratio and uterine ERα, progesterone receptor, and androgen receptor protein levels were not altered.]

The authors concluded that the decrease in ERα protein after genistein treatment may have been due to hydrolysis or to extended retention of nuclear receptor. They attributed the increase in progesterone receptor to a direct action of genistein on ERα and believed genistein exerted much of its action in this system through ERα in spite of its greater affinity for ERβ. Although they acknowledged the statistically significant decrease in androgen receptor protein, they questioned the biologic significance of this finding inasmuch as androgen receptor message was not decreased and testosterone serum levels were not decreased. The authors noted that the large dose of genistein given in this study may have remained for a prolonged time under the skin of the animals, serving as a repository for continuous exposure over time. They also cited studies showing that a greater proportion of an injected than an oral dose of genistein remains free (unconjugated) and therefore biologically active. **[The Expert Panel noted that the data from this study demonstrate uterotropic effects following sc but not dietary exposure, thus supporting the hypothesis that the sc route of exposure impacts the absorption and metabolism of genistein, resulting in greater concentrations of free, bioavailable genistein. Genistein blood levels following dietary exposure were not measured in this study, but an earlier study in the same laboratory demonstrated higher levels of free genistein with sc versus oral exposure *(209)*. In the earlier study, total genistein concentrations in serum following dietary exposure to 250 mg/kg diet from conception to PND 21 was 1810 ± 135 pmol/ml (average percent free genistein was 7%) compared with 5558 ± 1434 nM total genistein in serum after sc injection of 500 mg/kg bw on PND 16, 18 and 20 (average percent free genistein was 27%).]**

Strengths/Weaknesses: A strength of both the dietary and sc injection experiments is that pregnant rats were fed an AIN-76A phytoestrogen-free diet. Injections were carried out at approximately the same time each day and necropsy time was controlled, which would help to control diurnal variability in hormone measurements. Post-pubertal animals (50- and 100-day-old rats) were sacrificed in the same phase of the estrous cycle (estrus). The authors verified that genistein would not interfere with the procedure used to measure serum 17β-estradiol. Appropriate controls were included in the Western blot analyses and immunohistochemistry experiments. Both total and free (unconjugated) genistein levels were measured in serum following injection of genistein. A weakness is that in both the sc injection and dietary portions of this study, only 1 dose level of genistein was used, which does not allow for evaluation of dose-response relationships within a given dosing paradigm. Dose volumes were not given. The genistein dose injected sc was 500 mg/kg bw, which is a high dose level. There was no evidence that the authors controlled for litter effects (in fact, the authors do not state how many pregnant dams/litters were used in this study). Statistical description was inadequate (post-hoc test[s] not identified). Data for uterine and body weights were presented as ratios (raw data not given). 17β-Estradiol did not decrease *ERα* mRNA concurrent with decreases in ERα protein levels; similarly, genistein did not increase progesterone receptor mRNA expression concurrent with increases in progesterone receptor protein levels; these discrepancies were attributed to the time post-

dosing at which samples were collected. The dose equivalent (mg/kg bw) of the 250 mg/kg diet dose level used in this study was not given.

Utility (Adequacy) for CERHR Evaluation Process: This study is of limited utility for comparing the effect of route differences on genistein effects on the uterus due to concerns about experimental design.

Kouki et al., 2003 ***(614)***, in a study sponsored by a grant from the Ministry of Education, Science, Culture and Sports of Japan, examined the effects of neonatal genistein sc injection treatment of female rats. Female Wistar rats were sc injected with sesame oil (n=10), 1 mg daidzein (DZ, N=10), 100 µg estradiol (N=10), or 1 mg genistein/day (n=9) **[purity not specified]** from PND 1 (day of birth) to PND 5. **[Based on the assumed body weight of 0.052 kg for a female weanling rat *(313)*, the dose was estimated at 19 mg/kg bw/day.]** Each treatment group was represented by rats from 2 or 3 litters, and rats from the same litter received the same treatment. Rats were checked for vaginal opening. Vaginal smears were examined from the day of vaginal opening through PND 60, when rats were ovariectomized. Ovaries were fixed in Bouin fluid and examined for corpora lutea. One to two weeks following ovariectomy, rats were sc implanted with 17β-estradiol-containing tubes, and behavioral tests were conducted to examine sexual behavior with male rats. Statistical analyses included Mann-Whitney U-test for vaginal opening data and ANOVA for sexual behavior data.

The mean day of vaginal opening in the genistein group (28 days; range 26–35 days) was significantly accelerated compared to the control group (35 days; range 33–38).). Estradiol treatment also accelerated vaginal opeining. Normal estrous cycles were observed in all rats of the control group and in most of the daidzein injected females, but in no rats in the genistein or the estradiol groups. In the genistein group, 6/9 rats displayed prolonged estrus and 3/9 displayed persistent estrus. Ovarian weights were significantly reduced by almost half in the genistein group compared to controls. Corpora lutea were present in 2/9 rats in the genistein group, none of the estradiol treated females and all rats of the control group. In sexual behavior tests conducted at 2, 4, and 6 days following implantation with 17β-estradiol, the lordosis quotient was significantly lower than the control value only on the third day of testing **[~95 in control group and 68 in treated group]**. All but 1 of the genistein-treated rats displayed lordosis response. In comparison to other compounds also examined in this study, the response to genistein group was similar to the response to 17β-estradiol, although reduction of lordosis response was greater in the 17β-estradiol group, which were defeminized as were male controls. Most results for daidzein were similar to controls. The study authors concluded, "These results suggest that genistein acts as an estrogen in the sexual differentiation of the brain and causes defeminization of the brain in regulating lordosis and the estrous cycle in rats."

Strengths/Weaknesses: A strength of this study is that female Wistar rats were exposed to genistein 1 mg/day sc on PND 1–5, a dosing paradigm that included administration during the neonatal period; however, the sc route is not as relevant to human exposure. A relatively broad assessment of female reproductive endpoints was conducted including vaginal patency, estrous cyclicity, ovarian weight, corpora lutea counts, ovarian histopathology, and sexually dimorphic behavioral tests (lordosis quotient). Rats were ovariectomized and given implants of 17β-estradiol prior to behavioral tests in an effort to control for inter-animal variability in exogenous 17β-estradiol levels. Repeated measures ANOVA was used for the behavioral tests conducted on days 2, 4, and 6 after 17β-estradiol tubes

were implanted. Another strength is the use of an estradiol injected positive control, along with male controls for the behavioral tests. A weakness of the study is that only 1 dose level of genistein was used, which does not allow for evaluation of dose-response relationships. There was no evidence that the authors controlled for litter effects (i.e., the authors state that all female pups within a litter received the same treatment, and 2–3 litters were used in one treatment group. If the litter was the unit of analysis, sample sizes would have been n=2–3, which is insufficient for many of the parameters discussed). Furthermore, the authors apparently used multiple comparisons for numerous endpoints (genistein verses control, genistein versus daidzein, etc.), with no indication that there was protection of the alpha level to prevent Type I errors. However without knowing how the paired tests were done it might be that the contrasts were corrected for multiple comparisons.

Utility (Adequacy) for CERHR Evaluation Process: This study is of limited utility in the evaluation process.

Lamartiniere et al., 1998 ***(615)***, funded by NIH and the American Institute for Cancer Research, examined the effects of prepubertal genistein exposure via injection on reproductive and developmental toxicity in female Sprague Dawley rats. On PND 16, 18, and 20, twenty females/group were injected with DMSO vehicle or genistein **[purity not specified]** at 500 µg/g **[mg/kg]** bw. **[The treatment route was not specified but assumed to be sc based on other studies conducted in this laboratory.]** At 9 weeks of age, fertility was evaluated by mating the treated females to untreated males for 3 weeks. One untreated male was used for 1 treated and 1 control female. After birth of the litter, the dams were separated from the pups and bred to a different male. The rats were bred a total of 3 times. In each breeding cycle, 16–20 dams gave birth to litters. Although the number of litters in the genistein group was slightly lower, the effects were not statistically significant. **[Procedures for statistical analysis were not discussed for any of the endpoints in this study.]** No differences were detected in the number of male and female pups in either treatment group. After the third breeding, the dams were weighed and killed. No effects on body or ovarian weight were noted, but uterine weight was significantly reduced **[by 16%]** in the genistein-treated rats compared to controls. Though the number of ovarian follicles tended to be higher in the prepubertally treated rats, there were no significant effects on number of corpora lutea or numbers of normal or atretic primordial, growing, or antral follicles. **[Methods for ovarian histology were not specified.]** Offspring from the third litter were evaluated for endocrine-related parameters. Evaluations were performed on 16 genistein-exposed and 19 control litters. Compared to controls, there was no effect on body weight or anogenital distance in offspring born to dams treated prepubertally with genistein. No treatment-related effect was detected on sexual maturity, as determined by age of testicular descent or vaginal opening. Changes in estrous cycling were not detected in 16 female offspring per group at 43–50 days of age. Prepubertal genistein treatment of dams also had no detected effect on body, ovarian, or uterine weight of 50-day-old female offspring or prostate or epididymal weight of 56-day-old male offspring. Study authors concluded that genistein was too weak an estrogen to cause endocrine and reproductive tract changes following prepubertal exposure.

Strengths/Weaknesses: A weakness of this study is that methods for the continuous breeding study were not fully discussed. The purity of genistein was not given, and dose solutions were not analyzed for concentration or verified for stability or homogeneity. The authors did not mention the use of phytoestrogen-free diet, suggesting the possibility of additional genistein exposure. The authors used

only one dose level of genistein, so dose-response relationships could not be evaluated, and the dose may have been sc, which is not relevant for human exposure. There were no details as to how pups were assigned to treatment groups, and no indication was given that the authors controlled for litter effects. The authors reported that the uterine weights of multiparous female rats exposed to genistein were lower than control rats; however, there was no mention as to whether the authors controlled for estrous stage at necropsy. Methods for statistical analyses were not identified.

Utility (Adequacy) for CERHR Evaluation Process: This study is of no utility in the evaluation process.

Lee et al., 2004 ***(616)***, supported by the Korea Science & Engineering Foundation and Korea Research Foundation, examined the effect of genistein by sc injection on calbindin-D_{9k} expression in immature rat uterus. Sprague Dawley rats were obtained at 18 days of age and fed a soy-free diet. A series of studies was conducted following an acclimation period **[duration of acclimation period and age of rats at start of dosing not specified]**. In a dose-response experiment, rats were sc injected with DMSO (negative control, n=3/group), 17β-estradiol (positive control, n=3/group) or genistein **[purity not reported]** 0.4, 4, or 40 mg/kg bw/day (n=5/group) for 3 days. Rats were killed 24 hours following the last injection. In a study to examine the effects of genistein over time, 18 rats/group were sc injected with DMSO or genistein 40 mg/kg bw/day for 3 days, and 3 rats/group were killed at 3, 6, 12, 24, 48, or 72 hours following the last injection. In a third study, 10 rats **[presumably 2/group]** were sc injected with ICI 182,780 before sc injection with 40 mg/kg bw/day genistein or 17β-estradiol for 3 days and killed 24 hours following the last injection. Uteri were removed and RNA was extracted for northern blot and RT-PCR analysis of calbindin-D_{9k} expression. Protein levels of calbindin-D_{9k} in uterus were also measured by Western blot. Expression of ERα and ERβ protein and progesterone receptor mRNA were examined in the time-response study. Data were analyzed by ANOVA, Kruskal Wallis test, and Dunnett's test for multiple comparisons.

In the dose-response experiment, calbindin-D_{9k} protein levels in uterus were increased 3-fold following treatment with genistein 40 mg/kg bw/day. The time-response study demonstrated that calbindin-D_{9k} mRNA expression was increased from 3 to 12 hours following exposure, and protein levels were increased from 3 to 48 hours following exposure; control levels were obtained for mRNA at 24 hours and for protein by 72 hours following exposure. Pretreatment of rats with ICI 182,780 completely blocked increases in calbindin-D_{9k} protein expression that were induced by both genistein and 17β-estradiol. Genistein had no detected effect on ERβ protein expression. ERα protein expression was increased at 3 hours and returned to control levels at 12 hours following exposure. Progesterone receptor mRNA levels were increased at 3 hours following exposure and returned to control levels by 6 hours following exposure. According to the study authors, this study demonstrated that genistein stimulated calbindin-D_{9k} expression via the ERα receptor in immature rat uterus.

Strengths/Weaknesses: Strengths include the well defined treatment during prepuberty, the reasonable number of animals, the use of 3 dose levels of genistein, 2 of which were within the range of human exposure levels, the comparison to 17β-estradiol, and the use of ICI 182,780 pre-treatment to confirm the estrogenic nature of the effects observed. An additional strength is examining the mechanism of genistein action by using an ER-antagonist. Finally the time course of increased calbindin after genistein treatment is useful. The study also provides mechanistic information about uterine responses of several select estrogen-responsive genes (calbindin-D_{9k}, ERα, ERβ and PR) to genistein.

Weaknesses include the sc dose route, low numbers of animals tested, and the examination of only short-term uterine effects.

Utility (Adequacy) for CERHR Evaluation Process: This study is of no utility in the evaluation process; it provides consideration of mechanism of action of genistein in a female reproductive tissue at the sensitive developmental time of prepuberty. The finding that genistein treatment increases ERα expression may be relevant when evaluating the genistein-associated risk of uterine cancer.

3.3.1.4.2 Postnatal - Male Rats (oral)

Fritz et al., 2002 ***(617)***, funded by DoD and NIH, evaluated the effects of dietary administration genistein initiated during postnatal life on prostate development in the rat. Sprague Dawley rats on an unspecified diet were bred when females were 9 weeks old. Litters were culled at birth to 10 pups **[sex ratio not specified]** and weaned on PND 21. After birth, dams were given the phytoestrogen-free AIN-76A diet. Offspring were weaned to this diet with the addition of genistein (98.5% pure) at 0, 250, or 1000 mg/kg feed **[ppm; ~0, 37 and 147 mg/kg bw/day in weanling rats, estimated using EPA assumptions *(313)*]**. The 250 ppm diet was said to produce genistein serum concentrations at the "high physiological" level, and the 1000 ppm diet was said to produce serum concentrations at the "extreme of those found in humans consuming soy products" **[serum levels were not obtained in this study, but reference was made to Fritz et al., 2002 *(212)*]**. Additional animals were given the AIN-76A diet with diethylstilbestrol 75 µg/kg feed **[ppb]**. Other animals were fed the AIN-76A and received sc testosterone 10 mg/kg bw/day, dihydrotestosterone 2 mg/kg bw/day, or an equivalent volume of the DMSO vehicle on PND 26–35. **[A data table implies that there were no injections in the animals given treated feed. The number of animals in each group is specified as 8 in the data table; it is not known how many litters gave rise to these 8 animals per treatment group.]** Offspring were killed on PND 35, and the dorsolateral prostate was dissected. The individual lobes (dorsal prostate and types 1 and 2 lateral prostate) were identified in fixed whole mounts for measurement of bud perimeter and main duct length. Measurements were made of dorsolateral prostate 5α-reductase activity, expressed as percent dihydrotestosterone formed from total androgens (testosterone + dihydrotestosterone), and mRNA for dorsal protein 1, a marker for prostate differentiation, was determined using RT-PCR followed by electrophoresis and expressed by comparison to β-actin. Serum testosterone and dihydrotestosterone were determined with a kit. Data were analyzed by ANOVA with post-hoc Dunnett's test. Because there was no difference between the group receiving untreated AIN-76A and the group receiving DMSO, the AIN-76A group values were taken as control values.

There was no detected effect of genistein at either exposure level on relative dorsolateral prostate weight, mRNA expression of dorsal protein 1, or serum testosterone or dihydrotestosterone. Bud perimeter of the type 1 lateral prostate lobe was decreased by 23% in the group exposed to genistein 1000 ppm, but no other effects of genistein at either exposure level on prostate morphology were detected. Diethylstilbestrol decreased relative weight of the dorsolateral prostate, decreased the perimeter of all three lobes, and decreased dorsal protein 1 mRNA. The androgen treatments had effects opposite to those of diethylstilbestrol. The activity of 5α-reductase was said to be decreased 10% by dietary genistein 250 ppm and 14% by dietary genistein 1000 ppm. **[Data were not shown, and the *P* value was given as <0.08.]** The authors concluded that dietary genistein may have little estrogenic effect due to the extent to which it is conjugated, by comparison to estrogenic effects reported in studies using genistein injections, which result in a lower rate of conjugation.

Strengths/Weaknesses: A strength of the study is that after delivery, dams and subsequently male rats on study were fed a phytoestrogen-free AIN-76A diet. Genistein was 98.5% pure. Multiple dose levels of genistein (250 and 1000 mg/kg diet) were used, which allowed a dose-response assessment. The authors looked at the effects of genistein on the developing testis following exposure from weaning (PND 21) to PND 35. Genistein exposures were reportedly within realistic ranges for humans and did not alter body weights or feed consumption in experimental animals. Genistein was administered in the diet, a relevant route of exposure. Genistein blood levels were not reported, although these values were recorded in a previous experiment using this exposure paradigm. The authors examined genistein effects on both prostate structural (bud size) and functional parameters (5α-reductase levels and dorsal protein 1 expression). RNA data were normalized to β-actin expression. The effects of genistein were contrasted against effects seen with other estrogenic and androgenic materials. A weakness of the paper is that there was insufficient experimental detail to fully evaluate the study. There were no analytical data provided to verify dietary concentrations of genistein, stability of genistein in feed, or homogeneity of diets. There was no indication whether the authors controlled for litter effects. There was no information on how weanling rats were assigned to different treatment groups or whether they were singly housed during the study. Exposures were identified as 250 and 1000 mg/kg diet without conversion to dose levels on a mg/kg bw basis (data on feed consumption were not provided). While the authors stated that body weights were not affected by this dosing paradigm, body weights of animals were not given at any time point. There was no indication whether the authors controlled for diurnal variation or necropsy stress when collecting samples for serum hormone measurements. Activity of 5α-reductase was expressed only as percent of mean control activity; the value for mean control activity was not given. For prostate bud perimeter measurements, it was unclear how the authors listed the sample size in study Figure 2 (i.e., n=18+37+28, which is the sample size for lateral prostate type 1, lateral prostate type 2, and dorsolateral prostate, respectively. It appeared that each of those areas was analyzed separately). Due to technical difficulties, dorsal protein 1, a marker of prostate differentiation, could only be analyzed using whole dorsolateral prostate (not individual lobes). The relevance of genistein exposure in rats during this peripubertal period to human infants fed soy formula was not discussed.

Utility (Adequacy) for CERHR Evaluation Process: This study is of no utility in the evaluation process.

Fritz et al., 2003 ***(618)***, funded by DoD and NIH, evaluated the effects of dietary genistein beginning at weaning on testicular development in rats. Sprague Dawley rats were bred and given the phytoestrogen-free AIN-76A diet. Litters were culled at birth to 10 pups **[sex ratio not specified]**. Offspring were weaned on PND 21 to the AIN-76A diet with the addition of genistein (98.5% pure) at 0, 250, or 1000 mg/kg feed **[ppm]**. The 250 ppm diet was said to produce genistein serum concentrations at the "high physiological" level, and the 1000 ppm diet was said to produce serum concentrations at the "extreme of those found in humans consuming soy products." **[Serum levels were not obtained in this study, but reference was made to Fritz et al., 2002** ***(212)*****. This citation was also used to support the statement that feed consumption and weight were not altered by the dietary treatments.]** Additional animals were given the AIN-76A diet with diethylstilbestrol 75 µg/kg feed **[ppb]**. **[The design and the apparent number of animals in each treatment group (n=8) is identical to Fritz et al., 2002** ***(617)*****, discussed above, in which prostate was investigated, leading to the possibility that the same animals were used in both studies.]** Animals were killed on PND 35 and testes harvested. One testis was sectioned and a middle section fixed in formalin,

embedded in paraffin, and stained with hematoxylin and eosin. An additional piece of the same testis was subjected to Western blot analysis for androgen receptor, EGF receptor, extracellular signal-related kinase, and phosphorylated extracellular signal-related kinase. Immunohistochemistry was performed on deparaffinated sections for androgen receptor, phosphorylated extracellular signal-related kinase, EGF receptor, and PCNA. DNA fragment end-labeling was used in sections from 3 rats/group to detect apoptosis in seminiferous tubules. The other testis was used for assessment of aromatase activity, measured as the release of tritiated water after conversion of testosterone labeled in the 1 position. RT- PCR was used to measure mRNA for aromatase. Testicular testosterone and 17β-estradiol were measured using RIA kits. Statistical analysis was by one-way ANOVA with post-hoc Tukey test.

No statistically significant effects of genistein exposure on testis weight, seminiferous tubule dimensions, percent apoptotic tubules, testicular histology, immunohistochemistry, or testicular testosterone or 17β-estradiol were detected. **[The authors state that testicular testosterone "tended to be greater" and 17β-estradiol "tended to be lower," with *P* values given as <0.546 and <0.793 for the comparisons. The Expert Panel is not convinced that intratesticular steroid hormones were shown to be altered by treatment. The authors identified androgen receptor protein as decreased by genistein, although not significantly so. The Expert Panel found a significant decrease on re-analysis of the authors' data, however. The values and *P* values appear in Table 104.]**

Testicular aromatase activity and mRNA expression (compared to β-actin) were described as significantly decreased in the high-dose genistein group. **[Data analysis by CERHR did not identify a statistically significant effect of genistein, as indicated in Table 104.]**

Table 104. The Effect of Dietary Genistein (PND 21–35) on Rat Testis Development (Fritz et al., 2003)

Dietary Genistein, ppm	*Percent of Control*		
	Androgen Receptor Protein	*Aromatase Activity*	*Aromatase mRNA Relative to β-actin*
0	100.0 ± 9.1	100.0 ± 10.4	100.0 ± 12.5
250	88.2 ± 4.6	81.6 ± 11.8	87.9 ± 13.0
1000	71.0 ± 7.0[a]	74.8 ± 6.7[b]	71.9 ± 8.0[b]
ANOVA overall *P*-value	0.03	0.20	0.24
Benchmark Dose[c], ppm			
BMD_{10}	355	440	368
$BMDL_{10}$	241	237	209
$BMD_{1\ SD}$	688	1243	1139
$BMDL_{1\ SD}$	431	618	590

n = 8/group.

[a] $P < 0.05$ compared to 0 ppm group by post-hoc Tukey test.

[b] Identified as different from control by authors. **[*t*-testing performed by CERHR.]** For aromatase activity, $P = 0.06$ and for aromatase mRNA, $P = 0.08$.

[c] See the footnote to **Table 95** for an explanation of the use of benchmark dose in this report.

From Fritz et al., 2003 *(618)*.

The authors concluded that dietary genistein did not cause effects on the developing testis as adverse as did injected genistein, reported in other papers, and indicated that the difference by route of administration may be due to the greater proportion of genistein that is conjugated after oral administration. The authors believed the increase in testicular testosterone and decrease in testicular 17β-estradiol were consistent with a decrease in testicular aromatase. **[The Expert Panel notes that none of these increases and decreases were verified by statistical analysis.]**

Strengths/Weaknesses: A strength of this study was that after delivery, dams and subsequently male rats on study were fed a phytoestrogen-free AIN-76A diet. Genistein was 98.5% pure. Genistein was administered in the diet, a relevant route of exposure. The authors looked at the effects of genistein on the developing testes following exposure from weaning (PND 21) to PND 35. Multiple dose levels of genistein (250 and 1000 mg/kg diet) were used, which allowed a dose-response assessment. The authors examined genistein effects on a variety of testicular parameters, including testicular weight, morphology, apoptosis in the seminiferous tubules, androgen receptor protein concentration and localization, and expression of *EGF receptor* and extracellular signal-regulated kinases (*ERK*). The effects of genistein were compared to effects seen with the estrogenic positive control diethylstilbestrol. Genistein exposures were reportedly within realistic ranges for humans and did not alter body weight or feed consumption in experimental animals. Genistein blood levels were reported, although the values were recorded in a previous experiment using this exposure paradigm. The authors reported that appropriate negative (normal serum) and positive (unspecified tissues) controls were included in immunohistochemistry experiments. In experiments to determine aromatase activity, appropriate controls were included (i.e., testicular homogenates, addition of unlabeled testosterone to test samples, background radioactivity determination). In initial experiments to determine testicular testosterone and 17β-estradiol levels, the percent recovery demonstrated that loss of radioactivity was not significant. A weakness of this paper is that there was insufficient experimental detail to fully evaluate the study. There were no analytical data provided to verify dietary concentrations of genistein, stability of genistein in feed, or homogeneity of diets. There was no indication as to whether the authors controlled for litter effects. There was no information provided on how weanling rats were assigned to different treatment groups or whether they were singly housed during the study. While the authors stated that body weights were not affected by this dosing paradigm, body weights of animals were not given at any time point. This also was true for aromatase activity and aromatase expression (mRNA), which were reported relative to control values without presenting the control data. Testes sections were fixed in formalin, which is not the best preservative for tissue histopathology *(619)*. The number of nuclei examined per tubule was not specified in apoptosis experiments. Changes in testicular testosterone and 17β-estradiol levels were not statistically different. The relevance of genistein exposure in rats during this peripubertal period to human infants fed soy formula was not discussed.

Utility (Adequacy) for CERHR Evaluation Process: This study is of no utility in the evaluation process.

Wang et al., 2009 ***(620)***, supported by the U.S. Department of Defense, examined whether genistein chemoprevention of prostate cancer is only effective in a specific window of development (neonatal/prepubertal only, adult only, or life-time), and the potential mechanisms of genistein chemoprevention in rats. Female Lobund-Wistar (L-W) rats were purchased from the National Cancer Institute and fed AIN-76A diet (Harlan Teklad) for two weeks prior to mating; the AIN76A diet contains less than 10 pmol isoflavones/g diet. Females were mated with L-W male rats and allowed to deliver their litters

[bedding not described]. Genistein (98.5% pure) was mixed in the diet at 250 mg/kg AIN-76A diet; in a previous study, this dietary genistein concentration resulted in serum genistein concentrations in rats similar to those of humans eating a traditional Asian diet high in soy, and protected against chemically-induced mammary cancer in rats. Male offspring were assigned to one of the following four feeding regimens: Group A) life-time AIN-76A diet (no genistein) as the Control group (n=23), Group B) 250 mg genistein/kg AIN-76A diet from birth until 35 days of age, then fed the AIN-76A control diet (neonatal/prepubertal exposure, n=27), Group C) 250 mg genistein/kg AIN-76A diet starting at 90 days of age−20 days after cancer induction−until 11 months of age (adult exposure, n=28), and Group D) 250 mg genistein/kg AIN-76A diet from birth throughout life (life-time exposure, n=30). Males were chemically castrated by daily oral administration (gavage) of 33 mg Flutamide/kg body weight for 16 days beginning at 50 days of age; prostate cancer was induced by N-methylnitrosourea (NMU) at 70 days of age, followed one week later by implantation of a silastic capsule containing 25 mg testosterone to stimulate mitosis and promote tumor growth. Rats were weighed every two weeks and palpated twice a week for prostate tumors beginning 7 months after NMU injection. At 11 months of age (9 months after NMU injection), the animals were killed and 2necropsied; tumor size, origin and spread were noted. Prostate glands and associated tumors were collected and cut into two equal portions; one portion was used for measurement of AR, phosphor-Akt and PTEN by western blot analysis and the other portion was used for histopathology, immunohistochemistry, and *in situ* apoptosis labeling analysis. Cell proliferation, apoptosis, AR, PTEN and Akt proteins were measured in the dorsolateral prostate (DLP) of intact males receiving AIN-76 diet only (control) and in rats exposed to genistein from birth until day 70 postpartum (after chemical castration, but before NMU tumor induction) **[it is not clear from the methods, but this appears to be a subset of Group D]**. Ki-67 protein (cell proliferation indicator) was measured in normal DLP tissues and poorly differentiated prostate tumors by immunohistochemistry; for apoptosis assessment, the TUNEL assay (apoptosis indicator) was performed. Statistical analysis of histological specimens used exact Cochran-Armitage Trend Test to determine significance. Mean values for cell proliferation index, apoptosis index and band densitometry of western blots from animals exposed to genistein were compared to controls using two-sample student *t* test assuming unequal variances, with subsequent multiple comparisons.

By 11 months of age, 56% of the control rats had prostate cancer lesions with most of these (43%) being poorly differentiated. 22% of the Controls had normal tissue at 11 months; the remainder had low- and high-grade PIN. There was a significant association between treatments, however only the life-time exposure group was significantly different from the controls. The genistein treatment for the life-time exposure group and the adult exposure group had statistically significant downward trends for poorly differentiated tumors when compared to the control group, but no trend was observed in the neonatal/prepubertal exposure group; the greatest effect was seen in the life-time exposure group. Cell proliferation (Ki-67) was significantly lower (by 52%) and apoptosis (TUNEL assay) was significantly greater (by 45%) in the DLP of normal tissue from the genistein-exposed groups. In poorly differentiated prostate tumors, the genistein groups had 44% lower cell proliferation and 55% greater apoptosis. Cell proliferations to apoptosis ratios were lower by 78% in normal DLP and 74% in prostate tumors for the genistein-exposed groups when compared to the control group. AR and phospho-Akt protein levels were determined to be significantly down-regulated by 62% and 32% respectively in the DLP of genistein-exposed rats compared to controls; but PTEN protein expression was not significantly altered. Phosphorylated Akt protein in prostate tumors was significantly lower (56%) in prostate tumors of L-W rats exposed to genistein when compared to the control animals.

PTEN protein expression in the prostate tumors was significantly up-regulated (41%) in genistein-treated animals. There was no significant change observed in AR protein expression in the prostate tumors of L-W rats exposed to genistein.

Authors' conclusion: Dietary genistein reduces the incidence of advanced prostate cancer induced by NMU in L-W rats during adult and life-time exposure, the latter being more effective. The regulation of AR/Akt/PTEN axis by genistein may be one of the molecular mechanisms by which it inhibits cell proliferation and induces apoptosis, thus providing evidence of roles of genistein in prostate cancer prevention and treatment.

Strengths/Weaknesses: Strengths of the study are the use of dietary exposure, inclusion of information on the diets used and their isoflavone content, and the use of different exposure durations to determine whether the timing of exposure to genistein affect incidence of prostate cancer. Weaknesses of the study are the lack of assessment of a dose-response, no calculation of the actual dose of genistein received by the animals over the course of the study, and the stability of genistein in the diet was not determined. There was no measurement of serum genistein levels in the present study although it was indicated that based on a previous study the level of genistein in the diet was considered to be similar to an intake expected with a traditional Asian diet high in soy. In general, the results lacked detail making the study difficult to interpret. For example, there were no representative images of the histopathology of the prostates−the main endpoint of the study. For the cell proliferation and apoptosis parameters, percentage changes were indicated in the text without any supporting numerical data. Similarly, representative images of Western blots were provided to demonstrate changes in protein expression, however, no quantitation or numerical data was provided.

Utility (Adequacy) for CERHR Evaluation Process: The study is of limited utility due to lack assessment of a dose-response, lack of clarity regarding actual dose received, and the limited data displayed in the results. The suggestion from the data that lifelong exposure genistein may reduce risk of prostate cancer is worthy of additional study.

3.3.1.4.3 Postnatal - Male rats (non-oral)

Atanassova et al., 2000 ***(621)***, supported by the European Center for the Ecotoxicology of Chemicals and by AstraZeneca, examined the effects of neonatal treatment with genistein via sc injection on pubertal spermatogenesis and long-term changes in the reproductive system of male rats. As part of this study, adult female Wistar rats were fed standard diets (15.5% soy meal) or soy-free diets (soy substituted by fishmeal and cereal content increased from 64% to 78%) for 3 weeks prior to mating and through mating, pregnancy, and lactation. Male offspring of rats fed soy-free diets were maintained on soy-free diets from weaning until termination. An unspecified number of males born to mothers on the soy-free diets received sc injections of genistein **[purity not specified]** 4 mg/kg bw/day in phosphate-buffered saline vehicle on PND 2−18. The dose was selected to represent exposure levels of total phytoestrogens in 4-month-old infants fed soy formula. A group of soy-free controls were treated with vehicle. Males from the soy-free control group were compared to males in the standard diet control group. Male rats treated with genistein were compared to soy-free controls. **[Total number of rats treated was not stated, but 7−14 rats/group were evaluated.]** On PND 18 and 25, rats were killed and testes were fixed in Bouin fluid. Testicular cell numbers and seminiferous tubule lumen formation were determined by standard point counting of cell nuclei. Apoptosis was assessed by DNA fragmentation

detected by *in situ* DNA 3′-end labeling. Spermatocyte nuclear volume as a fraction of Sertoli cell nuclear volume was calculated as "an index of spermatogenic efficiency." Plasma FSH and inhibin B were measured by RIA and enzyme-linked immunosorbent assay (ELISA) methods, respectively. In addition, mating and fertility were examined in adult rats (80–90 days old) by placing them in a cage with an unexposed female for 7 days. Statistical significance was determined by ANOVA.

Results and statistical significance for endpoints characterizing pubertal spermatogenesis in 18- and 25-day-old rats are listed in **Table 105**. The study authors noted that the increase in spermatocyte nuclear volume per Sertoli cell nuclear volume in rats fed soy-free compared to standard diets on PND 18 suggested that dietary soy retarded pubertal spermatogenesis. Administration of genistein to rats reared on soy-free diets reversed the increase in spermatocyte nuclear volume per Sertoli cell nuclear volume and also slowed lumen formation, reduced FSH levels, and increased the germ cell apoptotic index compared to soy-free diet controls. For parameters also assessed on PND 25, the only significant effect that remained was the increase in spermatocyte nuclear volume per Sertoli cell nuclear volume in soy-free compared to standard diet controls. Testis weights in adult rats (90–100 days old) from the soy-free group were significantly higher (8%) compared to rats in the standard diet group, and testis weight of rats in the genistein group were similar to those in the soy-free group. Two of 9 males in the genistein group did not mate, 1 of the matings did not result in pregnancy, and all pups of 1 litter died shortly after birth; statistical significance was not attained. Animals in the soy-free control group were not mated.

In a larger study reported in this paper, body weight, testis weight, and plasma FSH levels were compared in 24 litters from soy-free groups and 29 litters from standard diet groups. Male rats were evaluated at 90–95 days of age. Rats in the soy-free group had significantly higher body weights (5.7%) and testis weights (3.6%) and significantly reduced plasma FSH levels (11.1%). **[Relative testis weights were not reported.]**

Table 105. Effects of Neonatal Exposures to Soy-Free Diet or Genistein on the Reproductive System of Male Rats (Atanassova et al., 2000)

Effect	*Comparison*	
	Soy-Free Control compared to Standard Diet Control	*Genistein [a] (Soy-Free Diet) compared to Soy-Free Control*
Germ cell apoptotic index, PND 18	↔	↑
Germ cell apoptotic index, PND 25	↔	↔
Seminiferous tubule lumen formation, PND 18	↔	↓
Plasma inhibin B, PND 18	↔	↔
Sertoli cell nuclear volume/testis, PND 18	↑	↔
Plasma FSH, PND 18	↔	↓
Plasma FSH, PND 25	↔	↔
Spermatocyte/Sertoli cell nuclear volume, PND 18	↑	↓
Spermatocyte/Sertoli cell nuclear volume, PND 25	↑	↔

[a] Genistein (4 mg/kg bw/day).
↑, ↓, ↔ Significant increase, decrease or no effect.
From Atanassova et al., 2000 *(621)*.

The study authors noted that effects of genistein exposure were similar to those seen in rats treated with 1 μg diethylstilbestrol, but unlike diethylstilbestrol, genistein was not shown to affect all facets of pubertal spermatogenesis. For example, genistein only mildly affected testicular weight and increased Sertoli cell nuclear volume per testis. Low doses of diethylstilbestrol (≤1 μg) and high doses of weak environmental estrogens (octylphenol at 0.5 mg and bisphenol A at 2 mg) were found to advance spermatogenic development. The study authors concluded that "the presence or absence of soy or genistein in the diet has significant short-term (pubertal spermatogenesis) and long-term (body weight, testis size, FSH levels, and possibly mating) effects on males."

Strengths/Weaknesses: A strength of this study is that the authors took several important steps to control for litter effects. They repeated each experiment at least twice and considered only reproducible effects as treatment-related, they pooled data from different experiments and from the several control groups to determine the spectrum of changes due to chance (historical control data), and in the statistical evaluation, they used pooled variance for each parameter of the study as a whole to minimize false positive findings. Samples sizes appeared to be sufficient. Genistein-treated animals and their negative control group were maintained on a soy-free diet, while a concurrent control given the standard diet with 15.5% soy meal was also included. Pups were treated with genistein on PND 2–18, which coincided with the neonatal period. Genistein was administered at a realistic concentration (4 mg/kg bw/day), a level reported to be equivalent to total phytoestrogen intake by human infants consuming soy formula. The authors referenced previous studies where methods were validated. Baseline FSH levels were determined in hypophysectomized rats and inhibin was confirmed to be undetectable in castrated adult male rat plasma. By monitoring multiple time points, the authors were able to evaluate long term effects of neonatal genistein exposure. A weakness of this study is that Wistar rats were bred in the authors' own breeding colony (not commercially available). The purity of genistein was not given, and dose solutions were not analyzed for concentration, stability, or homogeneity. While using 2 different concentrations of genistein (standard diet and 4 mg/kg bw/day sc), the dose of genistein consumed in the diet was not specified, and there were differences in route of exposure; thus, dose-response relationships were difficult to evaluate. Pup blood levels of genistein were not reported. The authors provided minimal information on body weights throughout the study. Mating and fertility experiments could have been performed more effectively. Samples of testicular cross-sections varied from 5 to 14 rats/group with no explanation for this variability in sample sizes. Statistics were conducted by ANOVA comparing control and treated groups at each age. It appears as if the authors conducted multiple comparisons without adequate protection against type I error. It is difficult to discern whether the different testicular effects between male rats on the standard diet and the soy-free diet were related to the difference in soy content or other nutritional differences between the diets. Odum *et al.*, 2001 *(622)* reported that different rodent diets containing varying amounts of phytoestrogens can have centrally mediated effects on rodent sexual development, rather than affecting peripheral ERs. Effects from these diets are likely due to nutritional differences between the diets. The soy-free diet had numerous changes compared with the standard diet (i.e., soy meal in the diet was substituted by fish meal; maize gluten was added, and the overall cereal content was increased to 78% compared with 64% in the standard diet).

Utility (Adequacy) for the CERHR Evaluative Process: This study is of no utility in the evaluation process.

Fisher et al., 1999 ***(623)***, from the UK Medical Research Council, examined the effects of neonatal treatment with genistein by sc injection and other suspected estrogenic compounds on development of testicular excurrent ducts in Wistar rats. The primary focus of the study was to establish dose-response relationships for diethylstilbestrol. Neonatal male rats, the mothers of which were fed a soy-free diet during gestation and lactation, were injected sc with genistein **[purity not specified]** 4 mg/kg bw/day in phosphate-buffered saline plus gelatin. **[Days of treatment were not specified, but the report states that PND 10 and 18 occurred during the dosing period.]** Dose selection was based on the estimated intake of isoflavonoids by infants fed soy formula. Controls were fed soy-free diets and were treated with vehicle (soy-free control). A second control group was injected with the corn oil vehicle used for administration of the other compounds examined (vehicle control). Rats were killed at 10, 18, 25, or 75 days of age. Testes and epididymides were removed and fixed in Bouin fluid. Rats that were 35 days old or older were perfused with 0.9% saline and 0.01% heparin prior to removal and fixation of testes. Testes were weighed, embedded in paraffin, and sectioned. Immunostaining to detect aquaporin-1, a protein the expression of which was reduced after diethylstilbestrol treatment, was followed by staining with hematoxylin and eosin for histologic analysis. Data for testicular weight and epithelial cell height were analyzed by ANOVA. Because effects on PND 18 did not differ significantly between the soy-free and vehicle control group, except for testicular weight, the 2 control groups were pooled for analyses conducted after PND 18. **[The number of rats treated with genistein was not specified, but for most endpoints, 3–14 genistein-treated rats were examined per group and time period.]**

On PND 18, no significant difference in testicular weights was detected between the genistein group and soy-free control group but testicular weights were significantly higher in the genistein compared to vehicle control group. Testicular weights in the soy-free control group were significantly higher compared to the vehicle control group. In the genistein group, no significant effects on testicular weights were noted on PND 25, but testicular weights were marginally but significantly higher than controls on PND 75. Genistein treatment had no detected effect on aquaporin-1 immunoexpression or on efferent duct or rete testis morphology, as was noted for the control group, on all days examined (PND 10, 18, 25, and 75). A small but significant reduction in epithelial efferent duct cell height was observed in the genistein group on PND 18 but no effects were seen on PND 25 or 75. Effects similar to those observed in the genistein group were seen in groups treated with octylphenol and bisphenol A. Treatment of rats with diethylstilbestrol 0.0037–0.37 mg/kg bw/day resulted in dose-dependently reduced testicular weight, distension of the rete testis and efferent ducts, reduction of efferent duct epithelial cell height, and/or decreased expression of aquaporin-1. Effects were most pronounced on PND 18 and 25; some effects became less pronounced with time, while others persisted into adulthood. Similar effects were noted in animals treated with ethinyl estradiol and tamoxifen. Treatment with a GnRH antagonist did not affect most endpoints, with the exception of permanent reduction in testis weight and transient reduction in efferent duct epithelial cell height, suggesting that estrogenic compounds cause direct, as opposed to indirect, effects through hormonal changes. The study authors noted that magnitude and duration of adverse effects were comparable to estrogenic potencies reported in *in vitro* assays.

Strengths/Weaknesses: A strength of this study is that genistein was administered at a realistic dose level (4 mg/kg bw/day), a level reported to be equivalent to total phytoestrogen intake by human infants consuming soy formula; however, the sc route is not relevant to human exposure. Appropriate negative controls were used for the immunocytochemistry experiments, and immunostaining was evaluated in at least 3 animals per age and treatment on at least 3 occasions. A weakness of this study

is that Wistar rats were bred in the authors' own breeding colony (not commercially available). The purity of genistein was not given, and dose solutions were not analyzed for concentration, stability, or homogeneity. The authors used only 1 dose level of genistein, so dose-response relationships could not be evaluated. Pup blood levels of genistein were not reported. There were no details on how pups were assigned to treatment groups, and there was no indication that the authors controlled for litter effects. The authors did not provide any information on body weights during the study. The exact dose period used for the genistein exposure was not specified, although the text indicated that the pups were still on treatment on PND 10 and 18. The authors state "As the soy-free control data did not differ significantly from control animals in any parameters assessed at day 18 (except testis weight), for simplicity, at all other ages assessed, the data from soy-free control animals were pooled with 'normal' control data." Given that testis weight differed on PND 18, the validity of this assumption seems questionable. It is possible that soy-free control data differed from "normal controls" at other time points. Figure 1 of the study showed a significant difference in testis weight for genistein on day 18 and stated that the genistein group was compare to the soy-free control group; however, the text for day 18 stated that "the testis weights of genistein treated and soy-free controls did not differ significantly." Per the authors' admission, rete testis morphology was difficult to assess in an objective and quantifiable manner, particularly given that cross-sections from identical regions of the tissue must be assessed. The authors stated that only gross changes could be detected easily. It seemed unlikely that cross-sections of the rete testis (planes of section) were the same in controls and treated samples and it was unclear if cross-section differences may have affected the results. Sample sizes varied from 3 to 20 rats/group/time point, and no explanation was given for this large variability in sample sizes. Statistics were conducted by ANOVA, comparing control and treated groups at each age. It appears as if the authors conducted multiple comparisons without adequate protection against type I error.

Utility (Adequacy) for CERHR Evaluation Process: This study is of no utility in the evaluation process.

Williams et al., 2001 ***(624)***, supported by the European Centre for the Ecotoxicology of Chemicals, AstraZeneca, and the European Union, evaluated the effect neonatal treatment with genistein by sc injection on sex steroid receptors in rat seminal vesicles. Neonatal Wistar rats (n=11–18; birth=PND 1) were treated with sc injections of corn oil or genistein **[purity not specified]** 4 mg/kg bw/day on PND 2–18 **[inferred from reference to *(621)*]**. On PND 18, seminal vesicles were dissected and fixed in Bouin fluid or frozen. Immunohistochemistry was used to evaluate ERα, ERβ, androgen receptor, and progesterone receptor. Western blot analysis was used to confirm changes in receptor levels in the seminal vesicles of some animals. **[It is not clear whether genistein-treated animals were evaluated by Western blot; statistical methods were not discussed and may not have been used.]** No genistein-associated changes in seminal vesicle histology or hormone receptor levels were seen. The authors concluded that in spite of using high doses in this study, "weak environmental estrogens," including genistein, did not produce changes in hormone receptors or seminal vesicle structure. The lack of effectiveness of these weak estrogens was attributed to lack of suppression of androgen receptor. By contrast, the stronger estrogens diethylstilbestrol and ethinyl estradiol suppressed androgen receptor, induced estrogen and progesterone receptor, and reduced epithelial branching in the seminal vesicles.

Strengths/Weaknesses: A strength of this study is that genistein was used at realistic human exposure levels. Group sizes were sufficient (seminal vesicles collected from 11–15 animals/treatment group), although there was no indication that the authors controlled for litter effects. Experimental controls

were adequate. Specificity of the antibodies used for immunocytochemistry and Western blots was confirmed. Immunolocalization studies were repeated on 3–5 occasions using sections from at least 3 animals to ensure reproducibility. Scores for immunostaining were based on at least 6 animals in two separate experiments. Diethylstilbestrol served as a positive control (high dose) and exhibited a dose-response relationship (lower doses) against which immunostaining could be scored. A weakness of this study is the sparse experimental detail. Wistar rats were bred in the authors' own breeding colony (not commercially available). There was no mention that a soy-free diet was used in these studies. The purity of genistein was not given, and dose solutions were not analyzed for concentration, stability, or homogeneity. Because the authors used only one dose level of genistein, dose-response relationships could not be evaluated. Pup blood levels of genistein were not reported. There were no details on how pups were assigned to treatment groups, and there was no indication that the authors controlled for litter effects. The authors provided no information on body weights. Although immunocytochemistry changes in seminal vesicle steroid receptors were confirmed by Western blot for selected chemicals, data on genistein were not presented. There was no information about statistical analyses.

Utility (Adequacy) for CERHR Evaluation Process: This study is of no utility in the evaluation process.

3.3.1.4.4 Postnatal - Female and Male Rats (oral)

Nagao et al., 2001 ***(625)***, supported by the Japanese Ministry of Health and Welfare, administered genistein by oral gavage to neonatal male and female Sprague Dawley rats. Untreated dams were permitted to deliver naturally and on the day of birth (PND 0), pups were sexed and weighed and litters were randomly culled to 4 males and 4 females where possible. Litters of 8 pups or fewer were not reduced. Pups were gavaged with genistein **[purity not specified]** at 0, 12.5, 25, 50, or 100 mg/kg bw/day on PND 1–5. A positive control group, used only for terminal histology evaluations, was given ethinyl estradiol 2 mg/kg bw/day by gavage on the same days. Pups were reared by their own dams and weaned on PND 21.

There were 31 males from 7 litters in the control group, 25 males from 5 litters in the 12.5 mg/kg genistein group, 25 males from 5 litters in the 25 mg/kg genistein group, 28 males from 5 litters in the 50 mg/kg genistein group, 23 males from 6 litters in the 100 mg/kg genistein group, and 10 males from 5 litters in the ethinyl estradiol group. On PND 21, five randomly selected males from the control and genistein-treated groups were killed and necropsied. Testes were fixed in Bouin fluid, stained with hematoxylin and eosin, and examined with light microscopy. In the surviving males, timing of preputial separation was assessed beginning on PND 35 and males were cohabited with untreated females at 12 weeks of age. Cohabitation was permitted on a 1:1 basis for up to 2 weeks or until sperm were found in the vaginal smear. Males that did not produce evidence of copulation were re-mated with a different untreated female for up to an additional 2 weeks. Copulated females were killed on day 12 of presumed gestation and uterine contents inspected. Partners of non-pregnant copulated females were mated for up to 2 weeks with one additional female. At least 2 weeks after copulation **[or at 18 weeks of age; the paper describes terminal sacrifice using both designations]**, males were killed and blood collected for measurement of serum testosterone. Reproductive organs were weighed. Thawed cauda epididymis was homogenized in water **[freezing of the cauda is not described]**, and sperm concentration determined using an automated system. Reproductive organs were histologically examined by observers who were blind to treatment status. Statistical analysis of offspring data used ANOVA with post-hoc *t* test for parametric variables and chi-squared or Kruskal-Wallis with post-hoc

Fisher's Least Significant Difference test or Mann-Whitney *U* test for nonparametric variables. Litter of origin was considered in the statistical analyses.

There were 29 females from 7 litters in the control group, 25 females from 5 litters in the 12.5 mg/kg bw/day genistein group, 21 females from 5 litters in the 25 mg/kg bw/day genistein group, 21 females from 5 litters in the 50 mg/kg bw/day genistein group, 25 females from 6 litters in the 100 mg/kg bw/day genistein group, and 10 females from 5 litters in the ethinyl estradiol group. On PND 21, five females from each litter were killed and necropsied. Uteri and ovaries were fixed in 0.1 M phosphate-buffered 10% formalin, stained with hematoxylin and eosin, and evaluated by light microscopy. Surviving females were followed beginning on PND 28 for vaginal opening. At 7 weeks of age, females underwent daily vaginal lavage for monitoring of estrous cyclicity. At 12 weeks of age, females were cohabited 1:1 with untreated males for up to 2 weeks. Copulation was assessed by sperm in the vaginal lavage. Females not copulating within 2 weeks were mated with new untreated males for up to an additional 2 weeks. Copulated females were killed on day 12 of presumed gestation and uterine contents evaluated. Females that had not copulated were killed at 18 weeks of age for histologic evaluation of the uteri and ovaries. **[The text also says that non-pregnant females were killed at 18 weeks of age, and a data table shows 20 non-pregnant females killed at 18 weeks; however, copulated females should have been killed on day 12 after copulation.]** Statistical analysis of offspring data used ANOVA with post-hoc *t* test for parametric variables and chi-squared or Kruskal-Wallis with post-hoc Fisher's Least Significant Difference test or Mann-Whitney *U* test for nonparametric variables. Litter of origin was considered in the statistical analyses.

There were no clinical signs in any pups during the treatment period, and viability was similar in control and treatment groups. There was a decrease in male body weight at the 100 mg/kg bw/day genistein dose at all time points (PND 6, 14, 21, and weeks 5, 7, 9, and 18 after birth) and at the 50 mg/kg bw/day genistein dose at weeks 5, 7, 9, and 18 after birth. Week 18 body weights were also decreased in the 12.5 and 25 mg/kg bw/day groups at terminal sacrifice. Benchmark dose values for body weight are given in **Table 106**. There were no detected differences among groups of males in time to preputial separation, copulation, or fertility, or in number of implants or number of resorptions in sired pregnancies. There were no detected differences among males in serum testosterone, epididymal sperm concentration, or testicular histologic changes, although a 100 mg/kg bw genistein-treated male showed testicular atrophy. Epididymal weight was decreased in all genistein groups, with a mean ± SEM control weight of 0.98 ± 0.03 g and mean weights in treated groups ranging from 0.90 to 0.92 g. **[Using the power model and number of offspring, BMD_{10} was 217 mg/kg bw/day, the $BMDL_{10}$ was 92 mg/kg bw/day, the $BMD_{1\ SD}$ was 299 mg/kg bw/day, and $BMDL_{1\ SD}$ was 124 mg/kg bw/day for epididymis weight.]** No treatment effects on relative epididymal weight were detected.

There was a decrease in female weight at the 100 mg/kg bw/day genistein dose at all time points (PND 6, 14, 21, and weeks 5, 7, and 9), in the 50 mg/kg bw/day genistein dose group at weeks 5, 7, and 9, and in the 12.5 and 25 mg/kg bw/day genistein dose groups at week 9. Benchmark dose values for body weight are given in **Table 106**. There were no detected differences among groups in age at vaginal opening, days at each stage of the estrous cycle, or mean estrous cycle length. The proportion of females showing normal estrous cycles was decreased in all genistein-exposed groups compared to the control proportion of 21/24. The authors indicated that the reduction in proportion of females showing normal estrous cycles was not dose related. The lack of dose relationship appears due to a proportion of normally cycling females of 8/20 in the 100 mg/kg bw/day dose group compared to 3/13 in the 50 mg/kg bw/day dose group.

Table 106. Benchmark Doses for Each Weighing Interval in Nagao et al., 2001

Weighing Interval	*Benchmark Dose[a], mg/kg bw/day*							
	Male Offspring				*Female Offspring*			
	BMD_{10}	$BMDL_{10}$	BMD_{1SD}	$BMDL_{1SD}$	BMD_{10}	$BMDL_{10}$	BMD_{1SD}	$BMDL_{1SD}$
PND 6	74	26	485	104	70	50	106	73
PND 14	127	67	223	102	100	61	160	96
PND 21	158	74	283	103	100	69	113	81
5 weeks[b]	142	87	140	84	113	79	98	67
7 weeks	118	75	127	79	98	71	84	60
9 weeks	177	99	172	95	107	74	102	69
18 weeks	78	52	112	73	N.D.	N.D.	N.D.	N.D.

[a] See the footnote to Table 95 an explanation of the use of benchmark dose in this report. A power model was used. Doses are rounded to the nearest whole number.

[b] Postnatal weeks.

N.D. = Not determined.

From Nagao et al., 2001 *(625)*.

No effect of genistein treatment on the proportion of females copulating was detected, but there was a decrease in the proportion of copulated females that were pregnant in all genistein-exposed groups. **[Using the power model and number of offspring treated/group, the BMD_{10} for this endpoint was 20 mg/kg bw/day, the $BMDL_{10}$ was 15 mg/kg bw/day, the $BMD_{1\ SD}$ was 91 mg/kg bw/day, and $BMDL_{1\ SD}$ was 63 mg/kg bw/day.]** The number of implants per litter was decreased at 100 mg/kg bw/day. **[Using the power model and the number of offspring treated/group, the BMD_{10} for this endpoint was 64 mg/kg bw/day, the $BMDL_{10}$ was 35 mg/kg bw/day, the $BMD_{1\ SD}$ was 115 mg/kg bw/day, and the $BMDL_{1\ SD}$ was 79 mg/kg bw/day.]**

On histologic evaluation of ovaries on PND 21, each genistein group was said to show polyovular follicles, whereas the control group had no polyovular follicles **[there were no data on the proportion of genistein-treated females with this finding]**. Among the female rats that were necropsied at 18 weeks, atrophic ovaries were reported in 1/5 rats in the 50 mg/kg bw/day genistein group and 5/10 rats in the 100 mg/kg bw/day group. Of the 9 rats in the 100 mg/kg bw/day group listed in a study table, 8 showed hypertrophy of uterine luminal epithelial cells. The study authors noted that histological findings such as ovarian atrophy and hypertrophy of uterine epithelial cells and myometrium in genistein-treated females were consistent with results in females exposed to other estrogenic substances. Hypertrophy of corpora lutea was also described in the text as occurring in "many" rats in the 50 mg/kg bw/day or lower groups. **[The table in the paper lists 14 rats in 50 mg/kg bw/day or lower groups, of which 5 had more than "very slight" hypertrophy of the myometrium and 4 had hypertrophy of corpora lutea.]** Histologic changes, such as hypertrophy of corpora lutea, increased luminal epithelial cell numbers, and increased epithelial folds, were believed by the authors to represent pseudopregnant-like changes associated with increased prolactin, which was shown in another study *(211)* to be produced by genistein treatment of rats. The authors contrasted these histologic changes with the changes produced by ethinyl estradiol in this study and other estrogens in other studies. The polyovular follicles seen on PND 21 in this study also occurred in other studies with other estrogens, according to these authors.

Strengths/Weaknesses: Strengths of this study include a relevant route of exposure during the neonatal period, use of a positive control group, mating of females with proven breeders, allowance of 2 breeding periods, and blinded histopathological evaluation of reproductive organs. Weaknesses of the study included not specifying purity of genistein and lack of analytical characterization of dose solutions (e.g., concentration verification, stability, homogeneity). A phytoestrogen-free diet was not used in these experiments. **[Feed contained ≤2.1 mg genistein/100 g and ≤1.9 mg daidzein/100 g from PND 21 to adulthood, but feed consumption data were not reported.]** On PND 0, litters were culled to 8 pups, 4 males and 4 females whenever possible, using 3–5 males or 3–5 females per litter. This point was difficult to reconcile with pup numbers in some cases (e.g., in the 50 mg/kg bw/day male group, 28 pups were used from 5 litters, implying that some litters contained >5 males). Furthermore, it appeared that the male and female offspring in the same dose groups came from different litters (e.g., 25 males and 25 females were exposed in the 12.5 mg/kg bw/day dose group from 5 litters (50 pups total), but litters were culled to 8 pups per litter, which would equal 40 pups. Different litters of males and females must have been used. Obviously, pup assignments were not clear. Assuming litter-based analyses with an n of 5–7, sample sizes were insufficient for some endpoints, particularly endpoints with greater inherent variance (e.g., epididymal sperm concentrations). It would have been useful if the authors had given some detail as to why estrous cycles did not meet the "normal" criteria inasmuch as there was no significant difference in estrous cycle length or days in any phase of the estrous cycle in genistein-treated animals. There were few details given with respect to serum collection for hormone measurements; consequently, the Expert Panel cannot verify whether the authors controlled for diurnal variation, necropsy stress, etc. It is unclear why thawed cauda epididymis was homogenized in water when typically a medium containing detergent (e.g., Triton X-100) is used. Age at puberty onset was measured; however, the authors did not report body weights at puberty onset. This parameter may be of interest because an estrogenic material might be expected to accelerate vaginal opening, whereas decreased rate of growth (body weight effects) might be expected to delay puberty onset. Vaginal opening at the same age as control animals may mask an effect if it occurred in the presence of decreased body weight (e.g., high-dose females weighed 10% less on PND 21). Incidence of polyovular follicles at 21 days was not given. The authors did not report female body weights after 9 weeks, so it was difficult to determine whether body weight differences may have affected some reproductive parameters. It would have been useful if the authors had reported blood genistein levels in treated pups. The authors stated that the litter of origin was considered in statistical analyses and specified a number of parameters evaluated using the litter as the unit of analysis; however, it does not appear that all endpoints were controlled for litter of origin. For example, it does not appear that litter was the unit of analysis for estrous stage length. Study Figure 1 lists n values as 24, 20, 14, 13, and 20, which does not suggest litter-based analyses. Furthermore, reproductive performance data (study Table 3) did not use a litter-based analysis.

Utility (Adequacy) for CERHR Evaluation Process: This multigenerational study is of high utility in the evaluation process due to its thoroughness, blinded design, use of appropriate route of administration, consideration of appropriate outcomes, and use of a positive control.

3.3.1.4.5 Postnatal - Female and Male Rats (non-oral)

Lewis et al., 2003 ***(243)***, funded by UK Foods Standards Agency, treated neonatal male and female rats **[strain not indicated]** by both sc injection and gavage treatment to simulate lactational exposure to genistein. Targeted exposures were 4 and 40 mg/kg bw/day orally. Preliminary studies showed that it would be difficult to achieve the high dose using exposure through the milk of treated dams due to

the limited access of genistein to milk, so direct dosing of pups was planned. Subcutaneous dosing of pups on PND 1–6 was used (due to the difficulty of gavaging very young pups in large numbers) followed by gavage treatment of pups on PND 7–21. The sc doses equivalent to the targeted oral doses were determined to be 0.2 and 4 mg/kg bw/day based on AUC determinations; however, due to an error, the experiment was initially performed with the high sc dose on PND 1–6 equivalent to an oral dose of 20 mg/kg bw/day. A subsequent study was added in which the correct sc dose was tested for one of the endpoints (volume of the SDN-POA). The low-dose genistein regimen produced AUC values after a single sc or oral dose of 4.58–7.52 µg equivalents-hours/ml, and the high dose regimen produced AUC values after a single sc or oral dose of 38.3–56.8 µg equivalents-hours/ml.

The main study on general postnatal development used 60 time-mated rats allocated to 3 equal groups. Rats were allowed to deliver their litters. Pups were dosed as indicated above. **[It is implied that pups within the same litter were given the same treatments.]** On PND 5, litters were standardized to include 3 or 4 males and 3–5 females **[final litter size not given]**. Dosing with genistein or vehicle was continued to PND 21. On PND 22, one male and one female pup per litter were killed and serum was taken for FSH and LH and for testosterone (males only) and 17β-estradiol and progesterone (females only). Uterine weights were recorded. Surviving males were evaluated for age and weight at testicular descent. Pups were weaned on PND 29, at which time up to 2 males and 2 females/litter were retained to make up groups of 30 males and 40 females per dose. Age and weight at vaginal opening and preputial separation were recorded. Daily vaginal smears were obtained from 20 females from the time of vaginal opening until the second proestrus, at which time the females were killed and serum was taken for measurement of FSH, LH, 17β-estradiol, and progesterone. Males were killed at 13 weeks of age, and serum was collected for FSH, LH, and testosterone measurement. Epididymides, prostates, seminal vesicles, and testes were weighed. **[In the results section, it appears that some females and males were killed at 12 rather than 13 weeks.]**

A separate study using reproductive neuroendocrine endpoints was performed using 5 pregnant rats in each of 4 dose groups. Animals were allowed to litter, following which 5 litters each were treated with diethylstilbestrol, low-dose genistein, high-dose genistein, or carboxymethylcellulose vehicle. As in the previous study, the treatments on PND 1–6 were sc and consisted of diethylstilbestrol 10 µg/kg bw/day, genistein 0.2 mg/kg bw/day, genistein 2 mg/kg bw/day, or vehicle. Treatments on PND 7–21 were by gavage and consisted of diethylstilbestrol 10 µg/kg bw/day, genistein 4 mg/kg bw/day, genistein 40 mg/kg bw/day, or vehicle. On PND 22, 10–12 rats/sex/dose group were retained. The remaining rats were killed and uterine and testis weights were recorded. The retained animals underwent ovariectomy and orchidectomy on PND 22–24. Intra-atrial cannulas were inserted between PND 42 and 54 for repetitive blood sampling. Blood was sampled for LH every 15 minutes, beginning 15 minutes before a 50-ng/kg gonadotropin-releasing hormone (GnRH) iv bolus through 30 minutes after the bolus. Animals were then anesthetized and perfusion fixed through the aorta with 4% paraformaldehyde. Brains were removed and stored for at least a week in 4% paraformaldehyde. Coronal sections were stained with cresyl violet, and the volume of the SDN-POA was estimated from serial sections using an image analysis system. Data analysis was by ANOVA with post-hoc *t* test. After PND 1, pup weight was evaluated using ANCOVA with PND 1 weight as a covariate. Proportions were evaluated using the Fisher's exact test.

In the general postnatal development study, no effect of genistein exposure on clinical condition or pup body weight gain was detected. Anogenital distance was described as not influenced by treatment on

PND 2. There was said to be no "biologically significant difference" in anogenital distance between genistein- and vehicle-treated pups on PND 22 **[data were not shown]**. No treatment-related effects on hormone levels in serum on PND 22 **[data were not shown]** were detected. Uterine weight on PND 22 was increased in animals exposed to the high dose of genistein compared to the controls **[mean ± SD uterine weights estimated from figure: control 25 ± 2.5 mg (n=17); low-dose genistein 27.5 ± 5 mg (n=17); high-dose genistein 52.5 ± 7.5 mg (n=14). The figure used μg; the Expert Panel assumes that mg was meant.]** There were no differences in uterine weight in 12-week-old animals exposed to genistein during the lactation period. Vaginal opening was advanced a mean of 4 days in the high-dose genistein group compared to the control. There was no detected change in age at vaginal opening in the low-dose genistein group. Most females in the high-dose genistein group demonstrated persistent vaginal cornification, and serum progesterone was lower in adult animals in the high-dose genistein group compared to controls. In the low-dose genistein group, females had vaginal cytology consistent with normal cycling. High-dose females had lower body weights than control or low-dose genistein females from PND 57 until the end of the experiment. **[The difference estimated from a graph was ~15 g.]** There were no treatment effects on body weight or reproductive organ weights in males. **[Effects seen at the highest dose of genistein (increased uterine weight at PND 22, accelerated vaginal opening, vaginal smears with persistent cornification, decreased body weight at week 7, and decrease progesterone levels) were consistent with an estrogenic response.]**

In the reproductive neuroendocrine study, absolute and relative uterine weights were increased by diethylstilbestrol and by the high dose of genistein. **[Mean ± SD uterine weights estimated from figure: control 20 ± 5 mg (n=3); low-dose genistein 20 ± 5 mg (n=6); high-dose genistein 40 ± 10 mg (n=5); diethylstilbestrol 120 ± 10 mg (n=5). The figure uses g; the Expert Panel assumes that mg was meant.]** Relative testis weight was said to be reduced by diethylstilbestrol but not by genistein. **[Data were not shown; absolute testis weights were shown and did not appear to have been affected by any treatment.]** Neither basal nor GnRH-stimulated LH concentrations on PND 42–54 were affected by lactation-period treatment **[data not shown]**. The volume of the SDN-POA was greater in control males than control females. The low dose of genistein had no effect on SDN-POA volume in males or females. The high dose of genistein and diethylstilbestrol increased SDN-POA volume in females.

The authors concluded that the highest dose of genistein, designed to be equivalent to 40 mg/kg bw/day, produced estrogenic effects in terms of uterine weight and produced persistent estrus, probably through alterations in hypothalamic development with prevention of the LH surge. The low dose of 4 mg/kg bw/day, which they believed represented anticipated exposures in infants consuming soy-based formulas, was without detectable effects.

Strengths/Weaknesses: A strength of this study is that the authors conducted a relatively thorough assessment of reproductive function in male and female rats following neonatal (PND 1–21) exposure to genistein. Genistein was 98.3% pure. Two dose levels were used, which allows some assessment of dose-response relationships, although the study would have been strengthened if 3 or more doses levels had been assessed to better define the dose-responses to genistein. Sample sizes, which varied with endpoints measured, were sufficient, although it was not clear that the authors controlled for litter effects. The low-dose level (4 mg/kg bw/day) was selected because it is the estimated exposure level for infants fed soy formula. The authors included a sophisticated approach to assess toxicokinetics of

genistein to select the best dosing paradigm. In addition, plasma concentrations of genistein and its metabolites were measured after both sc and oral dosing. The authors used these data to determine approximately equivalent sc dose levels that would achieve similar genistein AUCs (bioequivalent doses) as orally administered doses of 4 and 40 mg/kg bw/day, which allowed the authors to use sc administration of genistein in neonatal pups on PND 1–6 because it is technically difficult to gavage this number of pups at such a young age; however, it is a weakness that the results were based on the kinetics of a single dose of genistein administered sc or by oral gavage on PND 7. It is also unfortunate that there was a dosing error for high-dose pups on PND 1–6. While it is not clear that the authors controlled for litter effects at all time points, data collected on PND 22 were from 1 pup/sex/litter; thus, the increase in uterine weights observed on PND 22 was controlled for litter effects. Diethylstilbestrol was used as a positive control for some endpoints. Statistical analyses were appropriate for endpoints and sample sizes, although there was no indication that the litter was the unit of analysis. A weakness was that there was no indication whether dose solutions were analyzed to verify dose level, stability, or homogeneity. The diet R&M No. 3 contained ~100–110 ppm genistein (per Special Diet Services Ltd., Witham, Essex). While the authors measured time of testes descent, results for this measure were not reported. It was unclear why blood hormone concentrations and uterine weights on PND 22 were analyzed with both the pup and the litter as the unit of analysis; the litter is the correct unit. Anogenital distance and relative anogenital distance results were not shown. In the HPLC data, it was interesting that the retention times for metabolites IV and I did not change between the plasma and milk matrices (19 and 27 minutes, respectively), whereas the retention time for metabolite II shifted from 23 to 22 minutes (metabolite III had a retention time of 22 minutes, raising the question of a possible typographical error for retention time or metabolite number). In study Figures 7 and 11, the *y*-axis scale was apparently misstated and should have read weight in mg (not g). There was a large difference in the SDN-POA area in the two studies illustrated in study Figure 12 (i.e., area in control males was ~0.07 mm^3 in study 1 compared to ~0.021 mm^3 in study 2). There was no assessment of histopathology of the reproductive tissues or fertility in the adult animals. The relevance of genistein exposure in rats during the neonatal period (PND 1–21) to human hypothalamic development in infants fed soy formula was not discussed.

Utility (Adequacy) for CERHR Evaluation Process: This study has limited utility in the evaluation process. The higher dose of genistein did have an effect on female SDN and uterine weights, findings that have been noted before. The high dose of genistein also promoted earlier vaginal opening, persistent vaginal estrous and resulted in lower progesterone levels in adults which suggest ovarian dysfunction. The information about genistein concentrations in plasma after various doses and routes of administration is also useful. The lack of effects on the endpoints relevant to male reproductive development is somewhat reassuring. However, the study would have been strengthened if assessment of histopathology and fertility had been included in the study design. Another limitation is the use of a sc injection route of administration during PND 1-6.

3.3.1.5 Rats: Pre- and Postnatal

3.3.1.5.1 Pre- and Postnatal - Female Rats (oral)

Awoniyi et al., 1998 ***(626)***, supported by NIH and the University of Colorado, evaluated the effects of pre- and post-natal exposure to genistein on female rats to 12 pregnant Sprague Dawley rats were fed genistein **[purity not given]** in an isoflavone-free diet beginning on GD 17. **[A schematic diagram**

showed treatment beginning on GD 10; however, the text indicated that animals were purchased at GD 10. The plug day was not given.] The concentration of genistein in the diet was 5 mg/kg feed (ppm). A control group (n=8) was fed the isoflavone-free diet without added genistein. The resultant pups were weaned on PND 21. **[Standardization of litters was not mentioned. Feed consumption was said to have been measured, but no data on feed consumption were reported, and genistein intake was not estimated for dams during the gestation or lactation periods. Birth weights and litter size were not reported, although the authors commented that there were no adverse genistein effects on length of gestation, litter size, or offspring survival.]** At weaning, the female pups from 4 litters in each group (28 control and 30 genistein-exposed pups) were killed, and serum 17β-estradiol, progesterone, luteinizing hormone (LH), and follicle-stimulating hormone (FSH) were measured by radioimmunoassay (RIA). Reproductive organs were weighed and evaluated by light microscopy with hematoxylin/eosin or hematoxylin/periodic acid-Schiff (PAS).

The pups from 4 of the remaining genistein-exposed litters were weaned to the same genistein-containing diet that had been fed to their dams. The other genistein-exposed pups were weaned to the control diet. All the remaining pups from the control group were weaned to the control diet. The age at vaginal opening was determined in all pups, and daily vaginal smears were evaluated for estrous stage. All offspring were killed in proestrus at or near PND 70 **[called PND 70 for simplicity]**. Trunk blood was collected for determination of hormones as for the pups killed on PND 21. Reproductive organs were removed and evaluated in a manner similar to that for rats killed on PND 21. Treatment effects were evaluated with ANOVA with post-hoc Scheffé test.

Average genistein intake (± SEM) was 32.8 ± 1.0 µg/rat/day during the first week after weaning and 53.0 ± 3.0 µg/rat/day during the second week. **[Given the mean weight of the rats killed at weaning (54 ± 1 g), the mean genistein consumption during the first week after weaning would have been 0.98 mg/kg bw/day. Genistein intake, shown in a graph, was estimated at about 100 µg/rat/day on PND 42 and 49, 70 µg/rat/day on PND 56, 90 µg/rat/day on PND 63, and 80 µg/rat/day on PND 70. Body weight was given only for the weight at termination near PND 70, 215 ± 3 g, giving an estimate of genistein intake of 0.37 mg/kg bw/day at the end of the experiment.]** At weaning, rats that had been exposed to genistein weighed less than control rats (mean ± SEM: 54 ± 1 g genistein-exposed and 58 ± 1 g control). The ovaries and uteri of PND 21 females weighed less than the organs in the control group **[estimated from a figure as 20 mg (genistein) compared to 25 mg (control) for the ovaries and about 200 mg (genistein) compared to 250 mg (control) for the uteri. Relative organ weight was not reported]**. Serum LH appeared to differ markedly on PND 21 (mean ± SEM: genistein group 1990 ± 964 pg/ml compared to control group 270 ± 15 pg/ml,), but statistical significance was not achieved due to the large variance **[*P*=0.127, *t* test by CERHR using n=4 litters per dose group]**. Serum FSH was not shown to differ by dose group in PND 21 rats. 17β-Estradiol and progesterone serum concentrations were markedly decreased in the genistein-exposed rats on PND 21 (mean ± SEM: 17b-estradiol 3.9 ± 1.7 pg/ml genistein compared to 36.6 ± 4.1 pg/ml control, progesterone 1.2 ± 0.6 ng/ml genistein compared to 12.8 ± 1.5 ng/ml control). Follicular atresia was described as "conspicuous" in genistein-exposed ovaries assessed on PND 21. Follicular atresia was also present in the control ovaries, but "to a much lesser extent." **[Quantitative methods were not used.]**

Compared to body weights of rats never exposed to genistein, PND 70 body weights were significantly lower in rats continually exposed to genistein and significantly higher in rats exposed to genistein

prior to PND 21 and the control diet thereafter (mean ± SEM: continuous genistein 215 ± 3 g, control diet only 240 ± 5 g, genistein/control diet 281 ± 6 g). No treatment-group differences on PND 70 in serum LH, FSH, 17β-estradiol, progesterone, or in ovarian or uterine weight were detected. Although quantitative measures were not used, the authors stated that both groups of rats with genistein exposure prior to PND 21 had more frequent follicular atresia than rats never exposed to genistein. Animals exposed to genistein continuously until PND 70 were described as having hyperplastic and hypertrophic epithelia of the rete ovarii in 3 animals and flattened epithelia (as though by cystic dilatation) in the remaining 2 animals. **[This reference to 5 animals in this treatment group (which started with 4 litters) is the only mention of how many individual animals were evaluated at PND 70 or at any other time.]** The authors concluded that intrauterine and neonatal exposure to genistein may adversely affect reproductive processes in adult female rats.

Strengths/Weaknesses: A strength of this study is that it used isoflavone-free chow. Weaknesses included use of only 1 dose level of genistein, unknown purity of genistein, and small numbers of animals/group (n=4). Data did not appear to have been analyzed on per litter basis. Body weights were decreased, but no data on feed consumption were presented. At weaning, organ weights were not related to body weights. The Expert Panel had little confidence in the reliability of the dose level determination in this study. This study was difficult to statistically evaluate. The numbers of animals used in the statistical evaluations are not cited in most instances. As a result, when there is a large difference in the means between the treatment groups, the accompanying standard errors are too large to declare the results statistically significant. Such results are most likely due to sample sizes being quite small and the variability across animals to be large (perhaps a single animal skewing the results in one of the treatment groups).

Utility (Adequacy) for CERHR Evaluation Process: This study is of limited utility in the evaluation process due to flaws in experimental design.

Hughes et al., 2004 ***(627)***, supported by EPA, examined the effects of gestational and lactational genistein exposure (via gavage treatment to the dam) on uterine organization in adulthood. A similar study was conducted with soy milk and is described in **Section 3.4.1.1** of the Expert Panel report. This study was conducted in Long Evans hooded rats that were fed a phytoestrogen-free AIN-93G diet in which the soy oil was replaced with corn oil. Four dams **[4 dams/group assumed]** were randomly assigned to groups treated with genistein **[purity not given]** in corn oil at 0 or 15 mg/kg bw. Two positive control diethylstilbestrol groups (0.5 and 5 µg/kg bw) were used, and one group was exposed to genistein 15 mg/kg bw + diethylstilbestrol 0.5 µg/kg bw. Dams were gavaged with the test compounds from GD 14 **[day of vaginal plug not defined]** to PND 21 (day of delivery=PND 1). On a mg/kw bw basis, the genistein dose was said to be 10–15 times the dose received through a traditional Asian diet. On a caloric basis, the diet was said to be equivalent to use of soybeans as the exclusive protein source. On PND 60, 8 female offspring/group were killed and uteri were fixed in 4% paraformaldehyde for a histomorphometry examination and immunohistochemical analyses for PCNA, ERα, and progesterone receptor. Statistical analyses included ANOVA and Kruskal-Wallis test. The individual pups rather than the litter were considered the statistical unit. The pup-based analysis was said to be used because intrauterine position of pups, which was not considered, was said to have a greater impact on variances of outcomes than differences between dams.

The only effect of genistein compared to controls that was detected was a significant **[~20%]** increase in progesterone receptor expression in glandular epithelial cells. No effects of genistein treatment on luminal epithelial cell height, uterine proliferation, ERα expression in luminal or glandular epithelial cells, or progesterone expression in luminal epithelial cells were detected. Results observed with administration of genistein in combination with the low dose of diethylstilbestrol were similar to results observed with genistein alone. Significant effects in the high- and low-dose diethylstilbestrol groups compared to the control group included increased proliferation of luminal epithelial cells and increased expression of progesterone receptor in glandular epithelial cells. Additional significant effects in the high diethylstilbestrol group included increased luminal epithelial cell height and increased ERα expression in glandular and luminal epithelial cells. As discussed in **Section 3.4**, exposure of dams to soy milk during the lactation period also increased expression of the progesterone receptor in uterine glandular epithelial cells of the offspring. The study authors concluded that exposure of developing rats to isoflavones within human exposure levels induces an effect in an estrogen-responsive uterine marker long after cessation of exposure. Concerns were noted because the progesterone receptor is involved in several reproductive processes.

Strengths/Weaknesses: A strength of this study is the use of phytoestrogen-free chow. Weaknesses include small numbers of litters/group (n=4), administration of a single dose level of genistein (15 mg/kg bw), and not considering the litter as the experimental unit.

Utility (Adequacy) for CERHR Evaluation Process: This study is of limited utility due to the small numbers of animals and the single dose level of genistein used.

3.3.1.5.2 Pre- and Postnatal - Male Rats (oral)

Dalu et al., 2002 ***(628)*****,** supported by NIEHS, FDA, and the Department of Energy, reported the effects of developmental exposures to dietary genistein on adult male Sprague Dawley rats. This study was performed as part of a larger multigeneration reproductive study. At least 28 days prior to mating, parental F_0 male and female rats were placed on a soy- and alfalfa-free diet to which genistein (>99% purity) was added at dose levels of 0, 5, 100, or 500 ppm **[mg/kg feed]**. Dietary analysis confirmed the lack of detectable genistein and daidzein in the basal diet and that genistein concentrations were within 10% of nominal levels. Within genistein-exposed F_1 and F_2 litters, half of the male pups were weaned to their parents' diet and half were weaned to the control diet. Each of 12 litters was used to produce 1 or 2 pairs of males, with a pair consisting of males weaned to different diets (genistein-treated or control). The 12 litters gave rise to 17 pairs of male offspring, which were evaluated on PND 140. Trunk blood was collected for measurement of serum testosterone and dihydrotestosterone by RIA. Ventral and dorsal prostates and testes were dissected and weighed, after which they were frozen for later Western blot analysis of ERα and ERβ. Tissues from 6–10 animals/group were evaluated for histologic change by light microscopy. Generation and dose were treated as fixed effects and litter as a random effect in the statistical analysis. Significant effects from the mixed procedure of SAS® were evaluated by *t* test adjusted for multiple comparisons.

Results are summarized in **Table 107**. The authors identified a decreasing trend in body weight in F_1 rats exposed to genistein until PND 140. The significant effects on body and seminal vesicle weights

identified in animals exposed to 5 ppm genistein were considered by the study authors as likely due to chance. There were no observed effects of treatment on reproductive organ histology. Serum testosterone and dihydrotestosterone showed an increasing linear trend in F_1 rats exposed to genistein until PND 140.

Table 107. Effects of Developmental Dietary Exposure to Genistein on Adult Male Rats (Dalu et al., 2002)

Endpoint	Dose Level, ppm			Benchmark Dose, ppm[a]			
	5	100	500	BMD_{10}	$BMDL_{10}$	$BMD_{1\,SD}$	$BMDL_{1\,SD}$
F_1 Genistein exposure discontinued at weaning							
Body weight	↔	↔	↔				
Organ weight							
Dorsolateral prostate	↔	↔	↔				
Ventral prostate	↔	↔	↔				
Seminal vesicles	↔	↔	↔				
Epididymides	↔	↔	↔				
Testes	↔	↔	↔				
Serum testosterone	↓12%	↔	↑28%				
Serum dihydrotestosterone	↔	↑65%	↔				
ERα							
Dorsolateral prostate	↔	↔	↔				
Ventral prostate	↔	↔	↔				
ERβ							
Dorsolateral prostate	↓32%	↓41%	↓43%	192	130	869	508
Ventral prostate	↔	↓52%	↔				
F_1 Genistein exposure continued until PND 140							
Body weight	↔	↔	↓7%[b]	506	Failed	520	Failed
Organ weight							
Dorsolateral prostate	↔	↔	↔				
Ventral prostate	↔	↔	↔				
Seminal vesicles	↔c	↔	↔				
Epididymides	↔	↔	↔				
Testes	↔	↔	↔				
Serum testosterone	↔c	↔	↑95%[c]	37	28	74	61
Serum dihydrotestosterone	↔	↑80%	↑218%[c]	37	18	181	83
ERα							
Dorsolateral prostate	↔	↓41%	↔				
Ventral prostate	↔	↔	↓26%	464	201	514	495
ERβ							
Dorsolateral prostate	↔	↔	↔				
Ventral prostate	↔	↔	↔				

Table 107 (continued)

Endpoint	Dose Level, ppm			Benchmark Dose, ppm[a]			
	5	100	500	BMD_{10}	$BMDL_{10}$	$BMD_{1\,SD}$	$BMDL_{1\,SD}$
F_2 Genistein exposure discontinued at weaning							
Body weight	↓3%	↔	↑3%				
Organ weight							
Dorsolateral prostate	↔	↔	↔				
Ventral prostate	↔	↔	↔				
Seminal vesicles	↔	↔	↔				
Epididymides	↔	↔	↔				
Testes	↔	↔	↔				
Serum testosterone	↔	↔	↔				
Serum dihydrotestosterone	↔	↔	↔				
ERα							
Dorsolateral prostate	↔	↔	↔				
Ventral prostate	↔	↔	↔				
ERβ							
Dorsolateral prostate	↔	↔	↔				
Ventral prostate	↔	↓20%	↔				
F_2 Genistein exposure continued until PND 140							
Body weight	↔[c]	↔	↔[b]				
Organ weight							
Dorsolateral prostate	↔	↔	↔				
Ventral prostate	↔	↔	↔				
Seminal vesicles	↔	↔	↔				
Epididymides	↔	↔	↔				
Testes	↔	↔	↔				
Serum testosterone	↔[c]	↔	↔				
Serum dihydrotestosterone	↔	↔	↔				
ERα							
Dorsolateral prostate	↔	↔	↔				
Ventral prostate	↔	↔	↔				
ERβ							
Dorsolateral prostate	↔	↔	↔				
Ventral prostate	↔	↔	↔				

↑, ↓, ↔ Statistically significant increase, decrease, or no change.

[a] See the footnote to Table 95 for an explanation of the use of benchmark dose in this report. The number of animals used in the benchmark dose calculations was the lowest number of the range given in the report or 12 if no range was given. A power model with unequal variances was used.

[b] Value is significantly less than the corresponding value for the group (within dose and generation) that discontinued genistein exposure at weaning.

[c] Value is significantly greater than the corresponding value for the same group (within dose and generation) that discontinued genistein exposure at weaning.

From Dalu et al., 2002 *(628)*.

The authors called attention to the genistein-associated depression of ERβ in the dorsolateral prostate. **[This effect was almost entirely restricted to the F_1 generation.]** They concluded that the "apparent down-regulation of this receptor by genistein may have implications for reproductive toxicity and carcinogenesis."

Strengths/Weaknesses: Strengths of the study include use of soy- and alfalfa-free chow, analysis of chow for genistein and daidzein content, determination of genistein stability in chow, use of 3 genistein doses (5, 100, 500 mg/kg feed), and use of 12 litters per treatment group. Other strengths included the cross-over experimental design to determine reversibility of effects, multigenerational exposure, and use of the litter as the experimental unit. A weakness was evaluation of only males. Although data were presented on F_2 animals, no data were presented on reproductive performance of F_1 males.

Utility (Adequacy) for CERHR Evaluation Process: This study is of limited utility based on assessment of a dose-response, evaluation of relevant endpoints, and use of sufficient numbers of animals. A limitation of the study for the evaluation of soy infant formula is that animals were treated during gestation, lactation, and after weaning, making it difficult to discern which effects may be due to treatment during lactation alone.

Fritz et al., 2002 ***(212)***, funded by the Department of Defense (DoD) and NIH, evaluated the effects of dietary genistein *in utero* and during postnatal life on the developing prostate in Sprague Dawley rats. Seven-week-old females were placed on a phytoestrogen-free diet to which genistein (98.5% pure) was added at concentrations of 0, 25, or 250 mg/kg feed **[ppm]**. After 2 weeks on the diet, animals were mated and allowed to litter. On PND 1, pup body weight and anogenital distance were determined and litters were standardized to 10 pups. **[Sex ratio after culling was not given. The number of offspring for most evaluations appears to have been 16/group in the animals exposed from gestation through PND 70; the number of litters or distribution of animals among litters**

was not indicated. For sex ratio, at least 8 litters and more than 80 offspring were said to have been evaluated for each group.] Pups were weaned on PND 21 to the diet assigned to their dams until the pups were killed on PND 70. Separate groups of male rats were fed the phytoestrogen-free diet with genistein added at 0, 250, or 1000 mg/kg feed **[ppm]** on PND 57–65. On PND 66–70, the phytoestrogen-free diet was given without added genistein and animals were gavaged once daily with genistein in sesame oil at 0, 22, or 88 mg/kg bw/day, which approximated the daily genistein dose of the 0, 250, and 1000 ppm dietary treatments. The gavage treatments were used in place of dietary treatments at the end of the experiment to control more precisely the amount and timing of exposure. Animals were killed 9 hours after the genistein dose on PND 70. In both experiments, the dorsolateral prostates were dissected and frozen for subsequent study. Tissues were also fixed in 4% paraformaldehyde, embedded in paraffin, sectioned, and stained with hematoxylin and eosin for light microscopy. Homogenized prostate was evaluated by Western blot for ER and androgen receptor. RT-PCR was used to quantitate *ER* and androgen receptor RNA in comparison to β-actin. Serum testosterone and dihydrotestosterone were determined by RIA **[source of serum not specified]**. Statistical analysis was by ANOVA with post-hoc Tukey test. **[It appears that the groups treated only as adults were added to include a 1000 ppm exposure level for further evaluation of effects on sex hormone receptors noted in the experiment with prenatal and lifetime exposures.]**

Animal weight and feed intake were not given, except for terminal body weights of 414–422 g. **[The estimates of 22 and 88 mg/kg bw/day used to determine the gavage doses corresponding to the 250 and 1000 ppm treatments in the second experiment suggest that feed intake was about 36 g/rat. This estimate appears reasonable to the Expert Panel.]** Serum total genistein on PND 70 was reported **[method of analysis not given]** to be 18–28 nM **[5–8 µg/L aglycone equivalent]** for animals not given genistein, 167 nM **[45 µg/L aglycone equivalent]** for animals fed 25 ppm genistein in the diet, 1785–1908 nM **[482–516 µg/L aglycone equivalent]** for animals fed 250 ppm genistein in the diet, and 9640 nM **[2605 µg/L aglycone equivalent]** for animals given 1000 ppm genistein in the diet. **[Values are means; single values represent dose groups used in only one of the two experiments, and ranges represent values obtained in the two different experiments. These serum values are also discussed in Section 2.]**

Exposure to genistein during gestation, lactation, and through PND 70 had no observed effect on sex ratio, male anogenital distance, age at testicular descent, or on body or reproductive organ weight at PND 70. No effects of treatment on reproductive organ histology were detected. Serum testosterone was increased by treatment in these animals. Values were 2.61 ± 0.15 ng/ml for the control group, 3.28 ± 0.20 ng/ml for the 25 ppm group, and 3.36 ± 0.29 ng/ml for the 250 ppm group ($P<0.05$ **[error not given, but appears to be SEM (and SEM is used elsewhere in the paper); n=8 males per dose group, number of litters or litter of origin not specified]**. Benchmark dose calculations for serum testosterone levels are listed in **Table 108**. Dihydrotestosterone was not significantly affected by treatment in this experiment. In the group of animals treated only as adults, testosterone was characterized by the authors as increased by genistein treatment, although there was no effect of treatment by statistical analysis. The serum testosterone values were 1.97 ± 0.23 ng/ml in the control group, 3.10 ± 0.41 ng/ml in the 250 ppm group, and 3.40 ± 0.70 ng/ml in the 1000 ppm group **[error not indicated but assumed to be SEM]**. Serum dihydrotestosterone was not shown to be affected by treatment in this experiment.

Table 108. Benchmark Dose Calculations for Serum Testosterone Concentrations in Male Rats Fed Genistein from Conception Until 10 Weeks of Age or from 8 to 10 Weeks of Age (Fritz et al., 2002)

Treatment Period	Benchmark Dose[a], mg/kg diet			
	BMD_{10}	$BMDL_{10}$	$BMD_{1\ SD}$	$BMDL_{1\ SD}$
Lifetime	149	69	327	163
8–10 Weeks of age	195	79	1119	584

[a] See the footnote to Table 95 for an explanation of the use of benchmark dose in this report. A power model was used; n = 8 offspring per dose group. The variance was assumed to be SEM, as reported in other parts of the paper.

From Fritz et al., 2002 *(212)*.

The effects on prostate androgen and ER are shown in **Table 109**. ERβ protein was not measured because a suitable antibody was not available. The authors concluded that ERα was the most sensitive of these receptors because mRNA was suppressed at a dietary exposure level of 25 ppm. The authors further concluded that the 2-week adult exposure had an effect on receptors similar to that of lifetime exposure, suggesting that if genistein consumption in soy foods protects against prostate cancer, it might do so with adoption of a high-soy diet in adulthood, rather than requiring lifetime adoption of such a diet.

Table 109. Androgen Receptor in Rats on PND 70 after Genistein Exposure (Fritz et al., 2002)

Genistein Treatment	*Percent Control Value*	
	mRNA for Receptor	*Receptor Protein*
Androgen receptor		
Diet of pregnant and lactating dam + offspring to PND 70		
25 ppm	70	Not reported
250 ppm	15*	Not reported
Diet PND 57–65, gavage PND 66–70		
250 ppm-equivalent	70*	68
1000 ppm-equivalent	66*	64
ERα		
Diet of pregnant and lactating dam + offspring to PND 70		
25 ppm	60*	Not reported
250 ppm	52*	Not reported
Diet PND 57–65, gavage PND 66–70 (ppm-equivalent)		
250 ppm-equivalent	56*	92
1000 ppm-equivalent	49*	45*
ERβ		
Diet of pregnant and lactating dam + offspring to PND 70		
25 ppm	70	Not reported
250 ppm	40*	Not reported
Diet PND 57–65, gavage PND 66–70 (ppm-equivalent)		
250 ppm-equivalent	54*	Not reported
1000 ppm-equivalent	60*	Not reported

Percent reductions were estimated from graphs and, if possible, confirmed by the text
n=8 animals per treatment
Litter of origin not specified for animals exposed during gestation and lactation.
*$P<0.05$ compared to control value by ANOVA with post-hoc Tukey test.
From Fritz et al., 2002 *(212)*.

Strengths/Weaknesses: Strengths of the study include use of phytoestrogen-free chow and standardization of litter size on PND 1. A weakness is that the numbers of dams/treatment group were not presented (at least 8 according to text). Only 2 dose levels of genistein were used (25 and 250 mg/kg feed). It was not clear if the litter was used as the experimental unit for lifetime exposure groups. No data were presented on effects of genistein during pregnancy (gestation length, weight gain). Feed consumption was apparently determined during 10 days of adult exposure, but no data were presented. Because feed consumption was not reported, genistein exposures are unknown; however, serum genistein levels were determined at PND 70. Only male offspring were examined. It was not clear how 16 male rats were selected to be followed until PND 70 and it is also unknown if the same 16 rats were followed.

Utility (Adequacy) for CERHR Evaluation Process: This study is of no utility in determining developmental effects due to lack of experimental details and limited usefulness of several of endpoints presented. The data may be useful in corroborating results from other studies.

Latendresse et al., 2009 ***(478)*** supported by an Interagency Agreement between the US FDA and the National Institute for Environmental Health Sciences, examined the effects of dietary exposure to ethinyl estradiol and genistein on the male rat mammary gland in multigenerational reproductive and chronic toxicity studies. Two studies of identical design were conducted–one with ethinyl estradiol (EE_2) and one with genistein. Weanling Sprague Dawley NCTR CD rats (Strain Code 23) were obtained from the NCTR breeding colony and placed on control 5K96 diet for three weeks before being assigned to study at six weeks of age. On PND 42 the F_0 animals were randomized into exposure groups. The EE_2 study animals received diets containing 0 (control), 2, 10 or 50 ppb EE_2 (purity > 99%); the genistein study animals received diets containing 0 (control), 5, 100, or 500 ppm genistein (purity > 98.5%). The doses for both studies were established from the results of prior short-term studies; the high doses were selected to avoid significant maternal toxicity but still induce mild reproductive tract lesions in the offspring. The base diet for the studies was an alfalfa- and soy-free rodent feed (5K96, Purina Mills, Inc, Richmond, IN) containing casein in place of the soy and alfalfa protein. The levels of genistein and daidzein in the control diet were ≤5 ppm each, consistent with the isoflavone intake of humans consuming typical Western diets **[bedding not described]**. The F_0 generation was exposed to the prepared diets from six weeks of age until termination; the F_1 and F_2 generations were exposed from conception through termination; the F_3 generation was exposed from conception through weaning on PND 21 and then placed on the control diet until termination; the F_4 generation was not directly exposed to the prepared diets. *Multigenerational Studies*: (n=35 animals/sex/group/generation). For the F_0 through the F_3 generation, male and female rats in the same exposure group were paired for mating when they were 10 to 12 weeks old. Animals were killed on PND 140 and complete necropsies were performed. *Chronic Toxicity Studies*: A subset of male pups from the F_1 and F_2 generation of EE_2 study were used to examine recovery in a 90-day feed study. In the F_1 generation, one male from each of 18 litters was killed on PND 50 and a littermate was killed on PND 90. For the F_2 generation, the pups were removed from exposure at weaning and continued on control diet until termination on PND 50 or PND 90. A different subset of male weanling F_1 and F_3 animals from the multigenerational studies were assigned to 2-year studies, three exposure durations were studied (n=50/sex/group/exposure regimen): continuous exposure from conception through 2 years (F_1C–F_1 generation), exposure from conception through PND 140 followed by control diet until termination (F_1T140–F_1 generation), exposure from conception through weaning at PND 21 followed by control diet until termination (F_3T21–F_3 generation). All surviving animals were killed after two years and complete necropsies were performed. *All Studies*: At necropsy the inguinal mammary gland and fat pad were removed, placed flat in a cassette, fixed in NBF and frontal sections were prepared for microscopic examination (this procedure is comparable to a whole mount preparation). Mammary gland alveolar and ductal hyperplasia were assessed and assigned severity scores; these data were analyzed within each generation with a Jonckheere-Terpstra (JT) test for monotonic dose trend and a Shirley-Williams test to compare dose groups to control. Mammary gland sections from the control and high dose groups were assessed as to the prevalence of lobuloalveolar and tubuloalveolar development and scores were assigned to each morphologic pattern; a Wilcoxon test was used to assess the effect of treatment on the score distribution. The proportion of animals in the control and high dose groups showing one or more patterns, and those having predominately one or the other type, were compared using a two-sided Fischer's Exact test. The effect of treatment on mammary neoplasm prevalence was assessed by the Poly-*k* test. Trend analyses were conducted using both a standard linear dose trend and a coded dose scale.

Ductal and Alveolar Hyperplasia, 90-day EE_2 Feed Study: Duct hyperplasia was the predominate EE_2 effect in near-pubertal male rats treated from conception to termination on PND 50; the high-dose mammary tissue resembled mammary gland tissue from virgin control females in a different study. However, by PND 90, EE_2 had induced significant alveolar hyperplasia in the male rats. *Hyperplasia and Neoplasia, 90-Day EE_2 Feed Study*: In the EE_2 F_1C (continuous EE_2 exposure) animals at PND 50, the incidence of ductal hyperplasia tended to greater (dose-related manner) with minimal alveolar growth. By PND 90, hyperplasia of the alveoli had increased in both incidence and severity compared with the PND 50 rats, and duct hyperplasia was only marginally higher. In the F_2T21 rats (EE_2 exposure ended at PND 21), ductal and alveolar responses at PND 50 were similar to those observed in the continuously exposed F_1C generation, except that both incidence and severity of the mammary ducts were lower in the high dose group of the F_2T21 generation compared to the F_1C rats. By PND 90, the F_2T21 rats showed significantly less mammary gland hyperplasia when growth of both ducts and alveoli were combined compared to the rats that had been continuously exposed to EE_2. *Mammary Gland Evaluation at PND 140, Multigenerational Studies*: Mammary gland hyperplasia (alveolar and ductal) in rats exposed to EE_2 occurred with positive linear dose-related trends in F_0 through F_3 generation males, and there were significantly greater hyperplastic responses in some treated groups compared with the same-generation controls–the response was greatest in the F_1 and F_2 generations. In the F_0 (exposed from PND 42 to termination on PND 140) and the F_3 generations (exposed from conception through weaning), the mammary gland effect in males was much weaker, a significantly higher incidence of hyperplasia was only seen in the high dose. The genistein effects were very similar to EE_2 except the response was generally weaker. Positive linear dose trends occurred in the F_1 through F_3 generations; again, the response was greatest in the F_1 and F_2 generations (exposed from conception through termination on PND 140). Responses in the F_1 and F_2 generations were similar with the mid and high dose groups having significantly more mammary gland hyperplasia than controls (see **Table 110** for the incidence of mammary hyperplasia in the multigenerational genistein feed study).

Table 110. Incidence and Severity of Mammary Gland Hyperplasia in Male Rats in a Multigenerational Reproductive Toxicity Genistein Feed Study (Latendresse et al., 2009)

Generation	Dietary Genistein Dose Level			
	0 ppm	*5 ppm*	*100 ppm*	*500 ppm*
F_0	[a]1/23 (1.0)[b]	3/24 (1.3)	2/23 (1.5)	5/24 (1.6)*
F_1	1/26 (1.0)	1/24 (1.0)	5/25 (1.0)*	15/25 (1.9)***
F_2	2/24 (2.0)	0/25 (0.0)	8/25 (1.2)*	18/25 (1.6)***
F_3	4/24 (1.2)	2/25 (1.0)	6/25 (1.0)	8/23 (1.5)
F_4	4/25 (1.5)	4/25 (1.2)	6/25 (1.5)	6/24 (1.7)

The F_0 generation was exposed from six weeks of age until termination on PND 140.
The F_1 and F_2 generations were exposed from conception through termination on PND 140.
The F_3 generation was exposed from conception through weaning on PND 21 and then placed on the control diet until termination on PND 140.
The F_4 generation was not directly exposed to the prepared diets.

[a] The proportion of animals with mammary hyperplasia (number effected/number examined).
[b] Average severity grade of the lesion.
*Significantly different from the control group; $P \leq 0.05$.
***Significantly different from the control group; $P \leq 0.001$.

From Lantendresse et al., 2009 *(478)*.

Incidence of hyperplasia in the high dose group of the F_0 generation was significantly greater than the control group, but there was no linear response. In the F_3 generation, there was a dose-response trend, but none of the exposure groups were statistically different from the control. There was no mammary gland neoplasia in either multigenerational study and no pattern of feminization emerged among male rats treated with EE_2 or genistein.

Hyperplasia and Neoplasia, Two-Year EE_2 and Genistein Feed Studies: Some mammary gland hyperplasia was still evident in the two-year studies, but the overall severity of the response was considerably less than the animals examined at a younger age. EE_2 had a positive dose-related trend for alveolar hyperplasia in all three exposure regimens. However, only the F_1C and the F_1T140 showed a linear dose-response; both the mid and high dose groups were significantly higher incidence than the control. The incidence of ductal hyperplasia was significantly higher in the mid dose group for the F_1C and F_1T140 groups, but no linear dose-response was evident. F_3T21 showed a linear response trend for alveolar hyperplasia, the high dose was statically significant. The hyperplastic effects induced by genistein were present only in the F_1C and F_1T140 exposure regimens where positive linear dose-response trends were evident. However, only F_1T140 had a significantly higher incidence of alveolar hyperplasia in the high dose group (see **Table 111** for the incidence of mammary hyperplasia in the 2-year genistein feed study); no ductal hyperplasia was noted in any genistein exposure regimen.

In the EE_2 study, there was a significant positive trend in the incidence of mammary gland adenoma or adenocarcinoma (combined) in the F_3T21 males, but not in the incidence of these neoplasms in males of the F_1C or the F_1T140 exposure regimens where mammary gland hyperplasia was significantly greater. No fibroma or fibroadenoma occurred in control males in any of the exposure regimens in the genistein study, but two high dose F_3T21 males (genistein exposure from conception through weaning) had fibroma and two had fibroadenoma. These four neoplasms were considered unrelated to genistein exposure. There was no significant carcinogenic effect of treatment in the genistein two-year feed study.

Table 111. Incidence and Severity of Mammary Gland Hyperplasia in Male Rats in a Two-year Genistein Feed Study (Latendresse et al., 2009)

Generation / *Exposure*	*Dietary Genistein Dose Level*			
	0 ppm	*5 ppm*	*100 ppm*	*500 ppm*
F_1C	[a]1/44 (1.7)[b]	2/43 (1.5)	6/40 (1.7)	8/42 (1.4)
F_1T140	3/41 (1.7)	1/42 (1.0)	1/34 (2.0)	9/45 (1.4)*
F_3T21	4/39 (1.5)	5/43 (1.2)	6/41 (1.3)	6/41 (2.0)

F_1C were continuously exposed from conception through termination at 2 years of age.
F_1T140 were continuously exposed from conception through PND 140, followed by control feed until termination at 2 years of age.
F_3T21 were continuously exposed from conception through PND 21, followed by control feed until termination at 2 years of age.

[a] The proportion of animals with mammary hyperplasia (number effected/number examined).
[b] Average severity grade of the lesion.
* Significantly different from the control group; $P \leq 0.05$.

From Lantendresse et al., 2009 *(478)*.

Authors' conclusion: Results indicate that mammary gland hyperplasia in the male rat is one of the most sensitive markers of estrogenic endocrine disruption.

Strengths/Weaknesses: This study compared the effects of dietary ethinyl estradiol and genistein exposures on the mammary glands in rats. The multigenerational and chronic nature of the design seems generally relevant and appropriate to the goals of this project. Indeed, the analysis through F_4 is relatively unique. The general experimental design (materials, sources, diets) is reasonable and generally well described. The incorporation of several doses allows for exploration of potentially dose dependent effects. The NIH-31 diet is reasonable, although it is not immediately clear if the sources and concentrations of micro and macro-nutrients are as well controlled as is generally the case with AIN-based formulations. However, this is a widely used diet and the isoflavone content is very low (so this may not be a major concern).

Utility (Adequacy) for CERHR Evaluation Process: This study has limited utility for the evaluation.

Roberts et al., 2000 ***(629)***, supported by the University of Colorado and Colorado State University, evaluated the effects of dietary genistein during pregnancy and after birth on reproductive outcomes in male offspring. Pregnant Sprague Dawley rats were obtained on PND 10 **[plug day not specified]**. Dams were maintained on a isoflavonoid-free diet (AIN) with the addition of genistein **[purity not specified]** at 0 (n=8) or 5 (n=16) mg/kg feed **[ppm]**. The genistein exposure level was calculated to ensure ingestion of genistein at a level of at least 50 µg/kg bw/day, which the authors interpreted as equivalent to human intake. **[The authors cite Barnes et al., 1995 *(630)* for this estimate of human intake. The Barnes et al., citation is a review article that gives genistein intakes in humans as 20–80 mg/day in Asia and 1–3 mg/day in the US. For a 60 kg woman, these intakes are 333–1333 µg/kg bw/day in Asia and 17–50 µg/kg bw/day in the US (mostly consumed as genistein glycoside). Roberts et al., in the study under discussion, assume a 300 kg bw rat needs to consume a diet with a genistein level of 2.5 mg/kg feed to ingest genistein 50 µg/kg bw/day. They doubled this feed level to ensure that the target genistein intake would be reached. Actual feed consumption was recorded, according to the methods section, but was not reported in the paper. The EPA Biological Reference Value for a mature female rat is 0.08 kg feed consumed/kg bw/day *(631)*; therefore, a 300-kg rat would consume 0.024 kg feed/day. The use of genistein 5 mg/kg feed would, under these circumstances, result in a daily genistein intake of 120 µg/kg bw (all aglycone).]**

The treated or control diets were given to dams from GD 17 until weaning on PND 21 at which time 8 of the genistein-exposed litters were given control diets, and the other 8 litters were given the genistein diet. **[Only male offspring were studied; no mention is made of whether litters were adjusted to include a uniform number of males prior to weaning.]** Pups from 4 litters in each treatment group were killed for evaluation on PND 70, and the pups from the remaining 4 litters were killed on PND 130. Testes were obtained for histologic examination and spermatid counting, serum was obtained for radioimmunoassay of LH and FSH, and pituitaries were obtained for quantification of RNA for the β-subunit of FSH and LH. Statistical analysis was by 1-way ANOVA with post-hoc Scheffé test. **[There was no comment on whether litter of origin was considered in the analysis.]**

No treatment-related differences in offspring body weight were detected on PND 21 or 70. On PND 130, both groups of genistein-exposed offspring weighed 11–15% less than the control offspring.

Testis weight on PND 130 was 14% lower in animals exposed to genistein only prenatally and during lactation compared to control animals; animals exposed to genistein during prenatal life, lactation, and after weaning did not demonstrate a statistically significant reduction in testis weight on PND 130 (mean ± SEM: control 1.83 ± 0.06 g, exposure prenatally, during lactation, and after lactation 1.72 ± 0.04 g, exposure prenatally and during lactation 1.58 ± 0.05g). Epididymal weight was decreased in both genistein groups compared to control at PND 130. No treatment-related differences in testicular spermatid count were detected at either evaluation point. At the end of the lactation period (PND 21), genistein-exposed offspring compared to control offspring had a decrease in serum LH (mean ± SEM 174 ± 15.7 pg/ml compared to control value of 531 ± 72.8 pg/ml) and testosterone (0.88 ± 0.11 ng/ml compared to control value of 1.47 ± 0.23 ng/ml). No difference in serum FSH was detected. No significant differences between groups in hormonal measures on PND 70 were detected. On PND 130, both genistein-exposed groups had a mean 6–14% decrease in serum LH compared to controls without a significant difference in serum testosterone concentrations. Pituitaries from genistein-exposed PND 21 offspring contained less RNA for the β-subunit of LH than did control pituitaries. No treatment-related differences were detected in pituitary RNA for the β-subunit of FSH at any age or for the β-subunit of LH at the older ages. The authors concluded that "*in utero* and lactational exposure of male rats to dietary genistein did not have any negative impact on the pituitary gonadotropin gene expression, serum FSH and testosterone levels, and spermatogenesis at adulthood…although there was a significant reduction in serum LH levels." They also indicated in the discussion that both groups of genistein-exposed offspring reproduced normally; this information was presented as an "unpublished observation."

Strengths/Weaknesses: Strengths of this study include use of a semi-purified chow and the dietary cross-over design used at PND 21. Weaknesses include use of only 1 dose level of genistein (5 mg/kg feed), small numbers of animals/group (n=4), and no examination of organ weights relative to body weight. It was not clear if the litter was considered the experimental unit in data analyses. The Expert Panel has little confidence in the dose level determinations for this study.

Utility (Adequacy) for CERHR Evaluation Process: This study is of limited utility due to the use of a single dose level.

Wisniewski et al., 2003 ***(632)***, supported by NIH, evaluated male Long Evans rats after prenatal and lactational exposure to genistein in the diet of the dam. Adult female rats were fed a soy- and alfalfa-free diet supplemented with genistein **[purity not specified]** at 0, 5, or 300 mg/kg feed (n=4/dose group). After 2 weeks on the assigned feed, the females were bred and maintained on their assigned diets through pregnancy and lactation. Feed consumption during the pregnancy and lactation periods was comparable among groups. Estimated genistein intake during pregnancy and lactation was negligible in the basal diet group. In the group given genistein 5 mg/kg feed in the diet, the estimated mean genistein intakes of the dams were 100–200 mg/kg bw/day during pregnancy and 200–500 mg/kg bw/day during lactation. In the group given genistein at 300 mg/kg feed, estimated mean genistein intakes were 6400–9100 mg/kg bw/day during pregnancy and 12,700–23,600 mg/kg bw/day during lactation. **[Based on feed intake rates reported by the study authors and genistein intake rates reported in another study with similar dosing *(633)*, it appears that the authors made an error in reporting units and that intake rates should be 2 orders of magnitude lower (e.g., 1–2 mg/kg bw/day during pregnancy and 2–5 mg/kg bw/day during lactation at 5 mg/kg feed; 64–91 mg/kg bw/day during pregnancy and 127–236 mg/kg bw/day during lactation at 300 mg/kg feed).]**

Litter size, pup weight, and sex ratio were assessed on PND 2. Maternal behavior was assessed by removal of all pups and placement of a random 4 pups (2 of each sex) at the end of the cage opposite the nest. Time to retrieval of the first and last pup was recorded. Pup anogenital distance was recorded once/week beginning on PND 2. Pups were weaned on PND 21 and males housed together by litter. Males were assessed on PND 40–45 for penile length, testis diameter, and balanopreputial separation. On PND 70, penile length was again measured and males were placed with hormonally primed ovariectomized females for evaluation of sexual function. After testing of sexual function, males were killed and reproductive organs weighed. Testicular sperm count was assessed in homogenized paired testes. Plasma from retro-orbital sinus blood was evaluated for testosterone. Results, analyzed by ANOVA, are summarized in **Table 112**. **[There was no post-hoc test indicated, and there was no indication of litter analysis for male parameters.]**

Benchmark dose calculations for reproductive organ weights and plasma testosterone levels are listed in **Table 113**. The mating trials showed a greater effect of the low-dose genistein exposure with only 4/12 males mounting and intromitting compared to 9/12 animals in the control and high-dose genistein groups. There were no animals ejaculating in either of the genistein groups compared to 4/12 males in the control group. No effect of genistein on sperm count was detected. The authors concluded that low-dose genistein had a greater effect on subsequent male reproductive function than high-dose exposure and wrote, "Because exposure to the low dose of genistein was sufficient to exert permanent alterations in masculinization, the impact of dietary phytoestrogen exposure on human reproductive development should be investigated."

Strengths/Weaknesses: Strengths of the study included use of a soy- and alfalfa-free diet, determination of feed consumption and genistein intake, and testing for mating capability of treated rats. Weaknesses included use of only 2 genistein dose levels (5 and 300 mg/kg bw), unknown source and purity of genistein, and the small number of animals (4/group). In addition, it was not clear if the litter was used as the experimental unit for statistical analyses, and body weight should have been used as a covariate in the AGD analyses.

Utility (Adequacy) for CERHR Evaluation Process: Due to the small numbers of animals used, this study has no utility for the evaluation.

3.3.1.5.3 Pre- and Postnatal - Female and Male Rats (oral)

Casanova et al., 1999 ***(634)***, from the Chemical Industry Institute of Toxicology (CIIT), evaluated male and female Sprague-Dawley rats after prenatal and lactational exposure to genistein in the diet of the dam and of the offspring after weaning. Bred female Sprague Dawley rats were obtained on GD 1 (the day after overnight cohabitation). Six pregnant animals per group were randomized to 1 of 4 diets: 1. a soy- and alfalfa-free diet in which casein and corn oil were used instead of soy meal, soy oil, and alfalfa meal; 2. the soy- and alfalfa-free diet with genistein **[purity not specified]** added at 20 mg/100 g feed (0.02% **[20 ppm]**); 3. the soy- and alfalfa-free diet with genistein added at 100 mg/100 g feed (0.1% **[100 ppm]**); and 4. the standard NIH-07 rodent diet, which contains 12% (by weight) soybean meal, 4% alfalfa meal, and 2.5% soy oil. HPLC showed genistein and daidzein to be undetectable in the soy- and alfalfa-free diet. The NIH-07 diet contained genistein 16.0 ± 1.6 mg/100 g feed and daidzein 14.4 ± 2.4 mg/100 g feed (mean ± SEM). **[Using the mean feed consumption reported in the paper and an estimated dam weight of 250 g, genistein intakes would have been 20 mg/kg bw/day for the 0.02% diet, 87 mg/kg bw/day for the 0.1% diet, and 16 mg/kg bw/day for the NIH-07 diet.]** Dams were permitted to litter and nurse their own young.

Table 112. Effects on Pregnancy Outcome and Male Offspring of Feeding Genistein to Rat Dams During Pregnancy and Lactation (Wisniewski et al., 2003)

Parameter	*Genistein Added to Diet, mg/kg feed*	
	5	*300*
Maternal/Litter Characteristics		
Gestation length	↔	↔
Litter size	↔	↔
Sex ratio	↔	↔
Mean pup weight	↔	↔
Latency to retrieve pups	↔	↔
Male Offspring Characteristics	↔	↔
Anogenital distance		
PND 2	↔	↔
PND 7	↔	↔
PND 14	↔	↔
PND 21	↔	**[↓13%]**[a]
Body weight		
PND 21	↔	↔
PND 40–45	↔	↓17%
PND 70	Not reported	Not reported
Testis length, PND 40	**[↓10%]**[a]	**[↓11%]**[a]
Testis width, PND 40	**[↓11%]**[a]	**[↓11%]**[a]
Preputial separation by PND 40–45	**[↓77%]**[a]	**[↓77%]**[a]
Penis length, PND 70	↔	↔
Prostate weight, PND 70	↔ (↑41%?)[b]	↑19%[b]
Testis weight, PND 70	↔	↔
Seminal vesicle weight, PND 70	↔	↔
Epididymides weight, PND 70	↔	↓11%
Epididymal fat weight, PND 70	↔	↔
Plasma testosterone, PND 70	↓53%	↓40%
Latency to mount	↔	↔
Latency to intromission	↔	↔
Mean number of mounts	↔	↔
Mean number of intromissions	↔	↔
Proportion mounting	↓60%	↔
Proportion intromitting	↓60%	↔
Proportion ejaculating	↓100%	↓100%

↑, ↓, ↔ Significant increase, decrease or no difference compared to control.

[a] Estimated from a graph in the published paper.

[b] **[The authors' table appears to be in error in indicating a lack of significant difference in the prostate weight of animals born to dams given genistein 5 mg/kg feed. The numerical mean prostate weight (0.52 g) was higher in this group than in the 300 mg/kg group (0.44) and the SEM (0.04) and sample size (n = 12) were the same in these 2 groups. ANOVA with post-hoc Dunnett's test performed by CERHR showed the prostate weight in the 5 mg/kg group but not the 300 mg/kg group to be significantly higher than the control (0.37 ± 0.04 g).]**

From Wisniewski et al., 2003 *(632)*.

Table 113. Benchmark Dose Calculations for Adult Reproductive Measures Following Gestational and Lactational Exposure of Rats to Genistein (Wisniewski et al., 2003)

Parameter	*Benchmark dose[a], mg/kg diet*			
	BMD_{10}	*BMDL*	*$BMD_{1\ SD}$*	*$BMDL_{1\ SD}$*
Prostate weight	481	139	563	286
Epididymides weight	296	149	291	104
Plasma testosterone	153	53.4	612	197

A power model was used; n = 4 litters per dose group.
[a] See the footnote to Table 95 for an explanation of the use of benchmark dose in this report.
From Wisniewski et al., 2003 *(632)*.

Pups were sexed by anogenital distance and weighed as same-sex groups within litters within 24 hours of birth. Litter weights were monitored every 3 days. After weaning on PND 21, dams were killed and uteri inspected for implantation sites using 0.5% ammonium sulfide. Two or three pups of each sex per litter were killed at weaning for determination of gonad weight in both sexes and uterine weight in females. The remaining offspring were group housed by sex and maintained on the same diet as their dams. Individual offspring weight was determined on PND 21 and every 3 days thereafter. On PND 13, males were evaluated for thoracic nipple retention. Puberty was determined by vaginal opening or preputial separation. Females were killed at vaginal opening, and males were killed on PND 56. Ovaries, uteri, testes, and ventral prostates were weighed. Comparisons were made between groups using ANOVA with post-hoc Dunnett's test. Both the litter and the pup were evaluated as the statistical unit.

Significant differences in females identified by the authors are shown in **Table 114**. No differences among treatment groups were detected in implantation sites per dam, live pups per litter, or litter weight at birth. Feed intake per dam and dam weight gain were decreased in the group fed the soy- and alfalfa-free diet supplemented with 0.1% genistein. Male offspring weight gain on PND 22–56 was also reduced significantly on this diet. **[Trend testing by CERHR showed a significant decrease in weight associated with the amount of genistein added to the soy- and alfalfa-free diet.]** No relationship was detected between treatment group and female offspring weight gain on PND 22–34. Anogenital distance in males was not affected by treatment group, but in females, anogenital distance was increased with the NIH-07 diet **[and arguably with the addition of 0.1% genistein to the soy- and alfalfa-free diet, see Table 114]**. Relative anogenital distance also was increased in females with the addition of genistein to the soy- and alfalfa-free diet. Age and weight at vaginal opening were advanced and uterine weight on PND 21 was increased with the addition of genistein 0.1% to the soy- and alfalfa-free diet.

No differences by treatment were detected in the proportion of males with retained nipples, in age or weight at preputial separation, or in the weight (absolute or relative) of the testis (PND 21 or 56) or ventral prostate (PND 56) when the litter was considered the experimental unit. When the individual offspring was the experimental unit, relative testis weight was reported to be increased with the addition of genistein 0.1% to the soy- and alfalfa-free diet **[per offspring data not shown, litter data are included in Table 115]**. Absolute weight of the ventral prostate was reported also to have been reduced in this group when data were analyzed on a per offspring basis **[per offspring data not shown, see Table 115]**. Benchmark dose calculations are listed in **Table 116**.

Table 114. Significant Findings in Female Offspring of Rats Given Genistein Through Diet (Casanova et al., 1996)

Parameter	*Soy- and Alfalfa-Free Diet + Genistein Concentration*			*NIH-07 Rodent Diet*
	0%	*0.02%*	*0.1%*	
Dam weight gain GD 1–21, g	183 ± 8	187 ± 12 (n=4)	158 ± 5[a]	182 ± 6
Feed intake/dam, GD 1–21, g/day	25.4 ± 0.93	25.1 ± 0.9	21.7 ± 1.0*	26.6 ± 1.2
Anogenital distance in females, mm	1.07 ± 0.03	1.05 ± 0.03	1.20 ± 0.04[c]	1.21 ± 0.05*
Relative anogenital distance, mm/g x 10^3	153 ± 8[b]	160 ± 5	180 ± 7*	168 ± 7
Age at vaginal opening, days	32.0 ± 0.3	32.1 ± 0.8	29.6 ± 0.7	31.6 ± 0.5
Weight at vaginal opening, g	113.9 ± 3.8	105.9 ± 7.9	93.3 ± 2.8*	114.5 ± 3.4
Uterine weight PND 21, mg	26.9 ± 1.3	24.2 ± 1.3 (n=5)	60.6 ± 5.2*	27.4 ± 0.7
Relative uterine weight PND 21, mg/g	0.513 ± 0.0222	0.484 ± 0.02 (n=5)	1.248 ± 0.137*	0.493 ± 0.012

Data expressed as mean ± SEM, n=6/group except where indicated.

**P*<0.05, ANOVA with post-hoc Dunnett's test according to the authors.

[a] According to the authors, the *P* value by Dunnett's test was≈0.05. **[ANOVA by CERHR shows an overall *P* value <0.05 but no significant differences of any treatment compared to the soy- and alfalfa-free diet with the Dunnett's post-hoc test. Post-hoc *t*-test, however, gives *P*=0.024 for the comparison of the third and first columns.]**

[b] **[Test for linear trend by CERHR significant at *P*<0.05 for the first three columns.]**

[c] The authors did not identify this anogenital distance as significantly increased, although the similarity to the anogenital distance in the NIH-07 group is evident. The lack of significance appears attributed to the use of the post-hoc Dunnett's test. The use of either post-hoc Bonferroni or Newman-Keuls tests shows a significant increase in anogenital distance in this group.

From Casanova et al., 1996 *(634)*.

Table 115. Body, Testis, and Prostate Weight in Male Offspring of Rats Given Genistein through Diet (Casanova et al., 1999)

Parameter	*Soy- and Alfalfa-Free Diet + Genistein Concentration*			*NIH-07 Rodent Diet*
	0%	*0.02%*	*0.1%*	
Body weight gain PND 22–56, g	288.7 ± 3.5[a]	278.8 ± 6.3	265.7 ± 9.1[b]	301.5 ± 3.7
Testis weight PND 21, mg	235 ± 6	236 ± 3	219 ± 6	253 ± 10
Relative testis weight PND 21, mg/g	4.25 ± 0.10	4.43 ± 0.07	4.53 ± 0.05	4.31 ± 0.05
Testis weight PND 56, g	2.88 ± 0.06	2.93 ± 0.07	2.81 ± 0.06	2.90 ± 0.12
Relative testis weight PND 56, g/kg	8.28 ± 0.14	8.76 ± 0.26	8.85 ± 0.28[c]	8.01 ± 0.38
Ventral prostate weight PND 56, g	0.289 ± 0.010	0.318 ± 0.021	0.249 ± 0.009[c]	0.320 ± 0.018
Relative prostate weight PND 56, g/kg	0.830 ± 0.028	0.950 ± 0.067	0.794 ± 0.040[c]	0.875 ± 0.043

Data expressed as mean ± SEM, n=6/group.

[a] **[Test for linear trend by CERHR significant at *P*<0.05 for the first three columns.]**

[b] *P*<0.05, ANOVA with post-hoc Dunnett's test according to the authors.

[c] **[These values were said to be different from the values in the first column when the individual offspring was taken as the experimental unit. Per offspring data were not shown. The number of offspring per treatment group was not given.]**

From Casanova et al., 1999 *(634)*.

Table 116. Benchmark Dose Calculations for Rats Exposed to Genistein During Gestation, Lactation, and Following Weaning (Casanova et al., 1999)

Parameter	*Genistein in Diet, % [mg/kg bw/day]*			
	BMD_{10}	*BMDL*	$BMD_{1\ SD}$	$BMDL_{1\ SD}$
Feed intake/dam, GD1–21	0.0786 **[74]**	0.0457 **[43]**	0.0695 **[65]**	0.0353 **[33]**
Male pup weight gain, PND 2–17	0.0879 **[82]**	0.0515 **[48]**	0.0819 **[77]**	0.0459 **[43]**
Female pup weight gain, PND 2–17	0.0874 **[82]**	0.0447 **[42]**	0.112 **[105]**	0.0548 **[51]**
Male pup weight gain, PND 22–56	0.136 **[127]**	0.0827 **[77]**	0.0719 **[67]**	0.0422 **[39]**
Female pup weight gain, PND 22–34	0.0775 **[73]**	0.0407 **[38]**	0.0638 **[60]**	0.0322 **[30]**
Male relative anogenital distance	0.125 **[117]**	0.0475 **[44]**	0.156 **[146]**	0.0667 **[62]**
Female relative anogenital distance	0.0582 **[54]**	0.0364 **[34]**	0.0574 **[54]**	0.0362 **[34]**
Relative testis weight, PND 21	0.183 **[171]**	0.110 **[103]**	0.0687 **[64]**	0.0410 **[38]**
Relative testis weight, PND 56	0.192 **[180]**	0.0864 **[81]**	0.126 **[118]**	0.0581 **[54]**
Relative uterus weight, PND 21[a]	0.00554 **[5]**	0.00345 **[3]**	0.0254 **[24]**	0.0185 **[17]**
Weight at vaginal opening	0.0583 **[55]**	0.0382 **[36]**	0.0626 **[59]**	0.0385 **[36]**

[a] Linear model was used.

[Genistein inake was estimated assuming a mean feed intake of 23.4 g/day (the mean of the 2 groups given treated feed) and a dam weight of 250 g.]

From Casanova et al., 1999 *(634)*.

The authors concluded that the soy- and alfalfa-free diet was capable of supporting normal pregnancy and offspring development, and that dietary levels of genistein comparable to the levels in the NIH-07 diet had "minimal effects, with the possible exception of a slight increase in the female [anogenital distance], on the parameters that we used to assess rat reproductive development during the perinatal period." They contrasted the lack of a uterotrophic effect with the NIH-07 diet with the findings of Boettger-Tong *et al.*, 1998 *(635)* that a diet containing 21 mg genistein and 14 mg daidzein per 100 g of rat feed produced a uterotrophic response in immature ovariectomized rats, indicating that the effects of genistein on uterine growth in the intact animal may be more complex than simple additivity with the effects of native estrogens.

Strengths/Weaknesses: Strengths of the study include the use of dietary exposure during gestation and lactation, measurement of genistein and daidzein in the soy- and alfalfa-free and the NIH-07 diets, report of the composition of the diets (manufacturer information), measurement of food consumption and determination of genistein intake. Anogenital distance measurements were corrected for body weight. Weaknesses of the study include the fairly small number of animals/group (n=6). The use of only 2 genistein dose levels, no evaluation of histopathology of reproductive tissues, no evaluation of fertility of the F_1 generation, and there was no measurement of circulating levels of genistein.

Utility (Adequacy) for CERHR Evaluation Process: This study is of limited utility for the evaluation because only 2 dose levels were used, the number of animals/group was low, and there was no morphological assessment of reproductive tissues The data may be helpful when considering the results of other studies.

Delclos et al., 2001 ***(239)***, supported by NIEHS and FDA, conducted a preliminary study designed to identify dietary dose ranges for a larger NTP multigenerational study. Female Sprague Dawley rats

were given a soy- and alfalfa-free diet beginning 1 week before breeding. The day a vaginal plug was detected was GD 0. On GD 7, females were randomized to receive genistein (>99% purity) added to the soy- and alfalfa-free diet at 0, 5, 25, 100, 250, 625, and 1250 ppm **[mg/kg feed]**. The dosed feed was administered until weaning of pups on PND 21 (day of birth=PND 1). Five litters per dose group were retained for evaluation. On PND 2, litters were standardized to 4 males and 4 females where possible. Fostering within dose groups was used where necessary but was uncommon (5 males ended up being fostered within dose groups). After weaning, offspring were kept on the same dietary treatment as their dam until the offspring were killed on PND 50. Dams were killed at weaning and serum genistein determined (reported in *(213)*; see Chapter 2). In-life evaluations included body weight, feed consumption, number of live and dead pups, live litter weight, sex ratio, gross malformations, anogenital distance on PND 2, and developmental landmarks, including vaginal opening and preputial separation. At necropsy on PND 50, selected organ weights were obtained. Uteri, ovaries, oviducts, and vaginas were fixed in Bouin fluid, embedded in paraffin, sectioned, and stained with hematoxylin and eosin. The right testis and epididymis were used for determination of homogenization-resistant testicular spermatids and epididymal sperm analysis. The left testis and epididymis were fixed in Bouin fluid, embedded in paraffin, sectioned, and stained with hematoxylin/PAS. Seminal vesicles, coagulating glands, preputial glands, and prostates were fixed in neutral buffered formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin. Clinical chemistry and hematology tests were evaluated in blood in 2 rats/sex/litter. **[No differences by treatment in clinical chemistry and hematology parameters were detected according to the authors; data were not shown.]** Comparisons among groups were made with ANOVA and ANCOVA with post-hoc Dunnett's test. Incidence and severity of lesions on histopathology evaluation were analyzed using the Jonckheere-Terpstra test with the Williams modification of the Shirley test for comparisons of genistein-dosed groups to the control.

Evaluation of the soy- and alfalfa-free diet by LC-electrospray MS showed a mean ± SD genistein concentration of 0.54 ± 0.31 ppm and a daidzein concentration of 0.48 ± 0.31 ppm. Genistein intakes of dams and offspring exposed to treated diets were estimated based on feed consumption (**Table 117**). Feed consumption and body weight were decreased in the pregnant dams at the highest genistein dose level (1250 ppm) based on pair-wise comparisons on GD 20 and 21 and based on a significant trend from GD 12 onward. A significant trend with dose for gestational feed consumption and body weight gain was also identified. **[Using a power function to model the dose-response relationship for dam body weight effect yielded a BMD_{10}[12] of 380 ppm and a corresponding BMDL of 242 ppm; the $BMD_{1\ SD}$ was 606 ppm, and the $BMDL_{1\ SD}$ was 403 ppm. When feed consumption was modeled in a similar manner, the BMD_{10} was 540 ppm and the corresponding BMDL was 384 ppm, the $BMD_{1\ SD}$ was 742 ppm, and the $BMDL_{1\ SD}$ was 510 ppm. The data were modeled assuming that the total number of dams evaluated were the ones delivering a litter as indicated in Table 3 of the study.]** There was no effect of genistein dose on body weight of the dam during the lactation period.

The 1250 ppm genistein diet was associated with a decrease in the proportion of plug-positive dams that delivered litters (5/10, compared to 9/10 or 10/10 in the other groups). No effects of genistein treatment on the length of gestation, litter size, proportion of live pups, or sex ratio were detected. Mean live pup weight/litter was decreased (non-significantly) by treatment with 1250 ppm genistein.

[12] See the footnote to Table 95 for an explanation of the use of BMD in this report.

Table 117. Genistein Intake Ranges of Rats In Multigeneration Study of Delclos et al., 2001

Dietary Genistein, ppm	Pregnant Dams (GD 7–parturition)	Lactating Dams (PND 1–14)	Pups After Weaning (PND 21–50)	
			Male	Female
5	0.23–0.38	0.21–0.90	0.48–0.66	0.50–0.68
25	1.39–1.97	1.29–4.33	2.56–3.53	2.44–3.43
100	3.58–7.72	3.26–17.76	8.16–13.12	9.60–13.96
250	10.86–18.65	10.81–48.73	24.19–35.45	26.04–36.56
625	27.75–39.31	29.59–116.53	57.84–82.02	62.29–85.47
1250	69.96–96.75	73.10–202.75	124.76–238.25	141.17–213.73

From Delclos et al., 2001 *(239)*.

[The BMD_{10} using a linear model was 1848 ppm, the $BMDL_{10}$ was 471 ppm, the $BMD_{1\ SD}$ was 6704 ppm, and the $BMDL_{1\ SD}$ was 1634 ppm; however, because there were no differences by pair-wise analysis, the benchmark dose analysis may not be appropriate.] Significant main effects of dose or significant linear dose trends were identified in males for delays in righting reflex, eye opening, ear unfolding, and incisor eruption and in females for righting reflect, eye opening, and ear unfolding. **[The BMD_{10} and BMDL values for these endpoints are shown in Table 117.]** In addition, eye opening and ear unfolding were significantly delayed on pair-wise comparison in the 1250 ppm dose group. Anogenital distance on PND 2 was not affected by treatment in either sex. Preputial separation was not affected by genistein treatment; vaginal opening showed a significant linear dose trend for advancement. **[Benchmark dose values for vaginal opening are given in Table 118.]**

Table 118. Benchmark Doses Using Developmental Landmarks for which a Main Effect of Dose or Linear Trend Was Shown (Delclos et al., 2001)

Landmark	Dietary Genistein, ppm							
	Male				Female			
	BMD_{10}	$BMDL_{10}$	BMD_{1SD}	$BMDL_{1SD}$	BMD_{10}	BMDL	BMD_{1SD}	$BMDL_{1SD}$
Eye opening	1085	927	614	409	1008	821	439	272
Ear unfolding	1216	1005	476	282	1353	812	974	587
Righting reflex	227	127	912	562	309	163	1118	640
Incisor eruption	1560	1074	668	453	Trend not identified			
Vaginal opening	N/A				1277	1252	1205	494

See the footnote to Table 95 for an explanation of the use of benchmark dose in this report.
A power model was used; n=5 litters per dose group.
N/A=Not applicable.
From Delclos et al., 2001 *(239)*.

Offspring body weight gain on pair-wise comparison was depressed in the 1250 ppm genistein group beginning on PND 14. Benchmark dose values for terminal body weight are shown in **Table 119** for male and female offspring. There were apparent treatment-related effects on the absolute or relative weights of some organs, based on significant main effects of dose or significant linear or quadratic trends. These effects **[and associated benchmark doses]** are summarized in **Table 119**.

Table 119. Benchmark Dose Calculations Using Body and Organ Weights for which a Main Effect of Dose or Linear Trend Was Shown (Delclos et al., 2001)

Body or Organ	Male				Female			
	BMD_{10}	$BMDL_{10}$	$BMD_{1\ SD}$	$BMDL_{1\ SD}$	BMD_{10}	$BMDL_{10}$	$BMD_{1\ SD}$	$BMDL_{1\ SD}$
Body	↓1247	1062	1148	789	↓1113	876	507	312
Liver, absolute	↓1682	1189	1421	889	Trend not identified			
Liver, relative	↑1288[a]	1253	1237	1038	↑1941	1240	836	531
Thymus, absolute	↓633[a]	456	632	435	↓907	620	739	487
Pituitary, absolute	↑952	572	954	579	No adequate model			
Pituitary, relative	↑521[a]	399[b]	430[b]	321[b]	Trend not identified			
Preputial gland, relative	↑425	267	791	511	N/A			
Ventral prostate, absolute	↓447[a]	340[b]	519[b]	373[b]	N/A			
Ventral prostate, relative	↓580[a]	411	674	456	N/A			
Uterus, absolute	N/A				No adequate model			
Uterus, relative	N/A				No adequate model			
Vagina, relative	N/A				↑114	609	1196	666

See the footnote to Table 95 for an explanation of the use of benchmark dose in this report.
A power model was used except where noted; n=5 litters per dose group.
↓,↑ Direction of trend.
[a] Statistically significant change in animals at the high dose (1250 ppm dietary genistein) compared to the control animals by Dunnett's test, $P<0.05$.
[b] Linear model was selected.
N/A= Not applicable.
From Delclos et al., 2001 *(239)*.

There were no detected effects on absolute or relative weights of the testis, epididymis, dorsolateral prostate, or seminal vesicle/coagulating gland. Absolute and relative prostate weight decreased with increasing genistein exposure with a 28% decrement in ventral prostate weight in the group exposed to 1250 ppm genistein in the diet. There were no detected alterations in ovarian weight with treatment. Absolute and relative uterine weight showed a significant quadratic dose-trend with an inverted U-shaped dose-response curve. **[The Expert Panel noted that the inverted U is due entirely to the response at 625 ppm dietary genistein, for which the variance was very large.]**

Histopathologic abnormalities were seen in the ovaries of the 1250 ppm group; abnormalities consisted of more numerous antral follicles in various stages of degeneration compared to the control ovaries. Corpora lutea were smaller and fewer in number in the 1250 ppm group and appeared not to regress at the normal rate. When follicle counts were performed in 5 sections from each ovary in 12–15 animals from each dose group, no differences by treatment in number of primordial, growing, and antral follicles were detected. Only normal follicles were counted, so the apparent increase in degenerating antral follicles in the high-dose group would not have been identified by this method. Uterine and vaginal histopathology in the high-dose group showed inappropriate combinations of changes reflecting estrus, metestrus, and diestrus. In the vagina, abnormal cellular maturation labeled as dysynchronous was seen in 9/15 animals in the 1250 ppm group and 4/15 animals in the 625 ppm group. The authors felt these changes were consistent with increased progesterone effect, consistent with failure of the corpora lutea to involute appropriately. Mammary glands showed proliferation of alveolar complexes in the 250, 625, and 1250 ppm groups. There were elements of alveolar hyperplasia in all dose groups, but the severity of the hyperplastic process was increased in the 1250 ppm group. Treatment with genistein (at 250 ppm and higher doses) significantly increased the incidence of renal tubule mineralization in female and male rats.

In males, there was significant hypertrophy of mammary alveoli and ducts at 25 ppm and higher, with an increase in hyperplasia at 250 ppm and higher. **[It is not clear that mammary gland hypertrophy is an adverse effect.]** Abnormalities of spermatogenesis were seen in animals from all dose groups, consistent with the peripubertal status of these animals, but the severity of the abnormalities was increased in the 1250 ppm group. No difference by treatment group in testicular sperm head counts or epididymal sperm counts were detected. An increase in chronic inflammation of the dorsolateral prostate was seen in the 1250 ppm group.

The authors concluded that the 1250 ppm dietary level was clearly toxic and that most of the linear trends identified in the study were due to the effects at this high-dose level. They indicated that a dose of 500 ppm would be selected as the high dose for a planned multigeneration study to further characterize the effects of dietary genistein on the reproductive system.

Strengths/Weaknesses: Strengths of this study include 6 genistein dose levels in chow, the use of soy- and alfalfa-free chow, measurement of genistein and daidzein concentrations in chow, determination of feed consumption and genistein intake, and measurement of serum genistein concentration on PND 50. Except for histopathology data, the litter was used as the experimental unit. Many appropriate endpoints were examined. Weaknesses of the study include the use of only 5 litters/group in the follow-up evaluation and the lack of assessment of reproductive capability. However, this was a very thorough study design for a dose range-finding study conducted to select dose levels for a multigenerational study this.

Utility (Adequacy) for CERHR Evaluation Process: This study is of limited utility for the evaluation based on the large number of dose levels used. The study was well-designed and executed with appropriate statistical analyses and endpoints. A limitation of the study for the evaluation of soy infant formula is that animals were treated during gestation, lactation, and after weaning, making it difficult to discern which effects may be due to treatment during lactation alone.

Ferguson et al., 2009 ***(636)***, supported by Interagency Agreement between the U.S. Food and Drug Administration and the National Institute for Environmental Health Sciences, and the Oak Ridge Institute for Science Education, examined the critical periods for the alteration in sodium solution intake caused by lifelong dietary exposure to estrogen-like endocrine disruptors in adult rats (seen in previous studies), and to establish the effects of lower dietary concentrations of genistein and nonylphenol. In this five-generation study, male and female Sprague-Dawley rats from the National Center for Toxicological Research Breeding Colony were received on PND 21 and placed on a soy- and alfalfa-free diet (5K96, Purina Mills) - the soy and alfalfa protein in this control diet were replaced by casein, and the soy oil was replaced by corn oil; all rats were maintained on wood chip bedding **[type of wood not stated]**. On PND 42, the F_0 rats were placed on one of the following diets (n=35/sex/group): 5K96 chow containing 0, 5, 100, or 500 ppm genistein (purity>99%), or 0, 25, 200 or 750 ppm nonylphenol (purity >95%); these doses approximated 0.0, 0.4, 8.0 and 40.0 mg genistein/kg/day and 0.0, 2.0, 16.0 and 60.0 mg nonylphenol/kg/day **[basis of dose selection not stated]**. F_0 rats consumed the experimental diets from PND 42 throughout the remainder of the study, the F_1 and F_2 generations were exposed from conception throughout the remainder of the study. The F_3 generation was exposed from conception until weaning on PND 21 when all rats in this generation began to consume the 5K96 control chow and remained on the control chow throughout the remainder of the study; the F_4 generation was never exposed to the experimental diets and consumed the 5K96 control chow throughout the study. Mating began at approximately PND 70 for the F_0–F_3 generations; rats were paired for mating within their diet groups. Assessment of sodium solution intake was performed for F_1-F_4 generations in the genistein portion of the study, and for the F_1 and F_2 generations of the nonylphenol portion: On PND 65-68, two water bottles were placed in each rats cage (n=10/sex/group); one bottle contained regular water, the other bottle contained 3.0% sodium chloride in water. Daily water intake was measured for each of the three days, and body weights were measured on PND 70. Water intake data were analyzed using repeated measures ANOVA with group, sex, and day as factors. PND 70 body weights were analyzed separately for each generation using ANOVA with group and sex as factors.

For the F_1 and F_2 generations, the 100 ppm and 500 ppm genistein diet groups weighed significantly less than the control on PND 70; for the F_1 generation, the 750 ppm nonylphenol group weighed significantly less than the control on PND 70. There was no effect of diet of the PND 70 body weights in the F_2 generation for nonylphenol, or the F_3 or F_4 generations for either compound. Sodium solution intake was higher in females than males, but only minimal affects attributed to either diet in any generation were observed; statistical significance was only achieved on test day 1 (PND 65-66) for the F_1 500 ppm genistein group and the F_3 100 ppm genistein group.

Authors' conclusion: The results indicate that the dietary concentrations necessary to increase adult sodium solution intake in rats are greater than 500 ppm genistein and 750 ppm nonylphenol and such effects do not appear to increase across generations.

Strengths/Weaknesses: Strengths of the study include the us e of dietary exposure, inclusion of information on the diet contents including estimates of genistein and daidzein levels, assessment of a dose-response using 3 dose levels of genistein, use of a multi-generation study design to determine the effects of exposure during different period of development. A weakness of the study is the focus on single endpoint of sexually dimorphic behavior−the consumption of 3% Na solution in adulthood. This endpoint was considered by the authors to be a sensitive biomarker of estrogen exposure based on previous studies using higher dose levels of genistein and other compounds considered to have estrogen-like properties. Since there was no positive control in the present study it is unclear as to whether the lack of effect of genistein at ≤500 ppm is an indication of a lack of estrogen-like effects of genistein on the brain during development or is a reflection of the lack of sensitivity of the of the endpoint measured.

Utility (Adequacy) for CERHR Evaluation Process: The study has limited value for the present evaluation due to the focused nature of the study.

NCTR *(637)* released its final report in 2008 of a multigeneration reproductive toxicity study with genistein in the diet in Sprague Dawley rats. A preliminary study was first conducted to determine doses for the main study and that study is described in this report under Delclos *et al.*, 2001 *(239)*. The main reproductive toxicity study *(637)* was conducted according to GLP. A 2005 preliminary report of this study was considered in the 2006 Expert Panel evaluation of genistein and soy formula. Results of the main study relevant to development are briefly summarized here.

Six-week-old Sprague Dawley rats were assigned to dose groups (n=35/sex/group) using a stratified randomized procedure that resulted in similar body weights among groups. The rats were fed 5K96, a soy- and alfalfa-free diet to which genistein (≥99% purity) was added at 0, 5, 100, or 500 ppm. Mean genistein levels in control diet were measure at 0.417 ppm. Genistein doses for the entire feeding period in males were estimated by study authors at 0, 0.3, 7, and 35 mg/kg bw/day, and genistein doses for the entire feeding period in females were estimated at 0, 0.5, 10, and 51 mg/kg bw/day. The authors also provided estimated doses for females during periods when they were not lactating (0, 0.4, 9, and 44 mg/kg bw/day) and during lactation periods (0, 0.7, 15, and 78 mg/kg bw/day).

F_0 rats were exposed from 6 weeks of age through gestation and lactation periods and up to 140 days of age. At 70–84 days of age, F_0 rats were mated until a vaginal plug was detected or for up to 2 weeks. Day of plug detection was designated as GD 0. Twenty-five dams, sires, and litters/group were selected to continue in the study. Females were allowed to litter, and the day of birth was designated PND 1. On PND 2, litters were culled to 4 pups/sex/litter. Animals within the same dose group were fostered if needed to maintain litter size, but fostered animals were not mated or included in analyses. No more than 2 pups/sex/litter were randomly selected for breeding in the next generation. Parameters evaluated in adult rats included body weights, feed consumption, clinical observations, time for mating to occur, percentage of mated females delivering litters, and vaginal smears for 10 days prior to necropsy. On PND 1, pups were sexed, weighed, and evaluated for viability. Anogenital distance was measured on PND 2 in 10 litters/group. Body weight gain was measured during the postnatal period. All male pups were examined for retained nipples on PND 14. Onset of puberty was monitored. Vaginal cytology was assessed in 1 female offspring/litter for 14 days following vaginal opening and was also evaluated for 10 days prior to necropsy.

The same procedures were conducted in F_1, F_2, F_3, and F_4 rats, but there were differences in times of exposures. F_1 and F_2 generations were exposed from weaning at 3 weeks of age through 140 days of age, including gestation and lactation periods. F_3 rats were exposed indirectly during prenatal development and during the lactation period but were not exposed following weaning at 21 days of age. F_4 and F_5 rats were not exposed to genistein at any point in their lives. The F_0–F_4 generations were killed at 140 days of age. Necropsy observations included examination of uteri for resorption sites in females who had vaginal plugs but did not litter, organ weights, and histopathology. In all dose groups, histopathological examinations were conducted in tissues with gross lesions, reproductive organs (preserved in Bouin fluid), mammary glands, and kidneys of female rats and males rats of the F_1 and F_2 generations. Histopathological examinations of other tissues were conducted in animals from the control and high-dose groups. Sperm counts, motility, and morphology were determined. Ovarian follicles were counted in 8 females/group/generation. Pups from the F_5 generation, were monitored during the lactation period and killed at weaning. F_5 pups were not subjected to necropsy or histopathological evaluations. Twenty-five litters were selected for each generation. Statistical analyses included ANOVA, ANCOVA, Dunnett tests, Holm adjusted independent *t tests*, Wilcoxon tests, Kruskal-Wallis tests, Jonckheere-Terpstra nonparametric test, Kaplan-Meier procedure, and Shirley test.

Developmental effects observed in the multigeneration study included decreased live litter size in the F_2 generation of the 500 ppm group. A significant linear exposure concentration trend for decreased live litter size was also reported in the F_1, F_2, and F_3 generations. Significant differences in pre-weaning pup body weights are presented in **Table 120**.

Table 120. Pre-weaning Pup Body Weights for F_1 through F_4 Generations (NCTR, 2008)

Generation	Genistein, ppm					
	5		100		500	
	Male	Female	Male	Female	Male	Female
F_1	↓	↔	↓	↔	↓	↓
F_2	↔	↔	↔	↔	↓	↓
F_3	↔	↔	↔	↔	↓	↓
F_4	↔	↔	↓	↔	↓	↓

↑, ↓, ↔ Significantly lower than or no difference from control.
From NCTR, 2008 *(637)*.

Genistein treatment, primarily at 500 ppm, was also associated with decreased body weight and body weight gain in rats of other age groups (discussed below and summarized along with other treatment-related effects in (**Table 121**). Dose-related reductions analyzed with body weight as a covariate in anogenital distance on PND 2 were observed in F_1 males and females of the 500 ppm group, in the F_2 females in the 500 ppm group, and in F_3 females in the 100 ppm group. Anogenital distance adjusted for bodyweight (AGD/ the cube root of body weight) was also reduced in F_1 females of the 500 ppm group. Vaginal opening was accelerated in F_1 and F_2 females and body weight at vaginal opening was decreased in F_1, F_2, and F_3 females of the 500 ppm group. Testicular descent was delayed in F_3 rats of the 500 ppm group. In the 2 weeks following vaginal opening, the number of abnormal estrous cycles characterized by reduced time in proestrus and extended estrous or diestrus was increased in F_1 rats of the 500ppm group and increased estrous cycle length was observed in F_1 and F_2 rats of the high-dose group.

Table 121. Treatment-Related Results Observed in a Genistein Multigeneration Study in Sprague Dawley Rats (NCTR, 2008)

Parameter	Dose in Feed, ppm			Benchmark Dose, ppm			
	5	100	500	BMD_{10}	$BMDL_{10}$	$BMD_{1\,SD}$	$BMDL_{1\,SD}$
Body Weight of Fmales at 13 Weeks of Age (prior to delivery)							
F_0	↔	↔	↓10.8%	412	333	306	240
F_1	↔	↓6.7%	↓20.5%	265	220	275	220
F_2	↔	↔	↓9.8%	501	431	487	327
Terminal Body Weights							
F_0, females	↔	↔	↓8.6%	515	416	309	241
F_1, females	↔	↔	↓13.8%	374	309	272	218
F_2, females	↔	↔	↓5.8%	700	508	516	368
F_1, males	↔	↔	↓5.6%	994	652	667	432
Total Pre-Delivery Body Weight Gain in Females							
F_0, from 6 weeks old	↔	↔	↓16.2%	281	224	336	259
F_1, from 6 weeks old	↔	↔	↓28.2%	193	154	341	262
F_2, from 6 weeks old	↔	↔	↓16.3%	362	250	368	248
Total Male Body Weight Gain Throughout Study							
F_1, from 3 weeks old	↔	↓5.3%	↓5.8%	1160	523	770	474
F_3, from 3 weeks old	↔	↔	↓4.7%	1046	514	763	502
F_3, from 6 weeks old	↔	↔	↓7.5%	713	467	675	434
Total Feed Consumption of Females before Delivery of Litters							
F_0, from 6 weeks old	↔	↔	↓8.8%	586	445	406	302
F_1, from 6 weeks old	↔	↔	↓9.8%	572	392	592	399
F_4[a], from 6 weeks old	↔	↔	↓6.9%	852	539	741	461
Body Weights of Pups at Birth							
F_1, male	↔	↓6.3%	↔	612	515	623	520
F_5, male[a]	↓6.2%	↓9.2%	↓6.2%	2171	920	1868	526
F_5, female[a]	↓8.2%	↓8.2%	↓6.6%	2624	526	2408	524

Table 121 (continued)

Parameter	Dose in Feed, ppm			Benchmark Dose, ppm			
	5	100	500	BMD_{10}	$BMDL_{10}$	$BMD_{1\ SD}$	$BMDL_{1\ SD}$
Body Weights of Female Pups during the Lactation Period							
F_1 on PND 14	↔	↔	↓11.8%	425	306	505	355
F_1 on PND 21	↔	↔	↓14.3%	342	262	396	295
F_2 on PND 14	↔	↓8.6%	↔	517	444	541	504
F_2 on PND 21	↔	↔	↓6.0%	508	410	521	492
F_3 on PND 21	↔	↔	↓9.0%	479	334	514	358
F_4 on PND 21[a]	↔	↔	↓7.1%	567	372	676	435
Body Weights of Male Pups During the Lactation Period							
F_1 on PND 14	↔	↓12.0%	↓14.6%	425	313	464	334
F_1 on PND 21	↓4.9%	↓11.0%	↓12.8%	496	353	495	345
F_2 on PND 14	↔	↔	↓7.7%	547	379	578	397
F_2 on PND 21	↔	↔	↓11.4%	436	335	393	294
F_3 on PND 21	↔	↓6.4%	↓10.5%	549	373	587	391
F_4 on PND 21[a]	↑8.0%	↔	↓6.5%	514	344	639	419
Body Weight Gain of Female Pups during Lactation Period							
F_1	↔	↔	↓15.5%	300	231	385	288
F_3	↔	↔	↓11.7%	422	279	472	329
F_4[a]	↔	↔	↓8.4%	508	332	670	432
Body Weight Gain of Male Pups during Lactation Period							
F_1	↔	↓10.9	↓14.8%	389	289	431	312
F_2	↔	↔	↓14.9%	336	266	338	260
F_3	↔	↔	↓14.1%	400	290	479	338
F_4[a]	↔	↔	↓7.0%	452	301	637	416

Table 121 (continued)

Parameter	Dose in Feed, ppm			Benchmark Dose, ppm			
	5	100	500	BMD_{10}	$BMDL_{10}$	$BMD_{1\ SD}$	$BMDL_{1\ SD}$
Number Male Rats/Number Treated with Mammary Alveolar or Ductal Hyperplasia							
F_0	3/24	2/23	↑5/24				
F_1[b]	1/24	↑5/25	↑15/25				
F_2[b]	0/25	↑8/25	↑18/25				
F_3[b]	2/25	6/25	8/23				
Number Male Rats/Number Treated with Renal Tubule Mineralization							
F_1	3/25	↑8/25	↑15/25				
F_2	1/25	↑4/25	↑6/25				
Number Male Rats/Number Treated with Renal Cysts							
F_1	3/25	0/25	↑3/25				
F_2	2/25	1/25	↑3/25				
Number F_1 male rats/ Number treated with kidney inflammation	15/26	19/25	↑22/25				
Number F_1 male rats/ Number treated with regeneration of renal tubules	6/25	8/25	↑19/25				
Live Pups Born							
Total F_1[c]	↔	↔	12.6%	382	199	610	483
Total F_2[c]	↔	↔	↓30.4%	154	121	376	285
Total F_3[c]	↔	↔	12.4%	486	317	513	475
Female F_2[c]	↔	↔	↓32.8%	134	101	463	335
Male F_2[c]	↔	↔	↓28.1%	457	130	510	430
Anogenital Distance							
F_1, male pups	↔	↔	↓5.6%	1174	578	819	398
F_1, female pups	↔	↔	↓6.9%	1039	511	596	335
F_2, female pups	↔	↔	↓4.2%	1767	520	678	360
F_3, female pups	↔	↓5.9%	↔	1294	703	667	357

Table 121 (continued)

Parameter	Dose in Feed, ppm			Benchmark Dose, ppm			
	5	100	500	BMD_{10}	$BMDL_{10}$	$BMD_{1\ SD}$	$BMDL_{1\ SD}$
F_1 Female anogenital distance ratio	↔	↔	↓5.6%	1227	507	793	392
Age at Vaginal Opening							
F_1	↔	↔	↓2.9 days	510	421	522	500
F_2	↔	↔	↓2.8 days	669	480	636	450
F_3	↓1.3 days	↔	↔	628	531	599	519
Body Weight at Vaginal Opening							
F_1	↓10.5%	↔	↓27.3%	280	162	500	367
F_2	↔	↔	↓18.9%	470	279	504	457
F_3	↔	↔	↓15.4%	462	288	921	562
Age at testicular descent, F_3	↔	↔	↑1.9 days	529	500	529	500
Number cycles with abnormal diestrous stage (following vaginal opening), F_1	↔	↔	↑1.0 cycle	888	416		
Number cycles with abnormal estrous stage (following vaginal opening), F_1	↔	↔	↑0.4 cycles	821	501		
Number cycles with abnormal diestrous or estrous stage (following vaginal opening), F_1	↔	↔	↑1.4 cycles	681	375		
Length of Estrous Cycle Following Vaginal Opening							
F_1	↔	↔	↑3.2 days	91	63	393	293
F_2	↔	↔	↑0.83 days	321	256	275	217
Number cycles with abnormal diestrous or estrous stage (before sacrifice), F_3	↔	↔	↑0.42 cycles	1061	488		

↑, ↓, ↔ Statistically significant increase, decrease or no effect.

[a]Animals received no direct or indirect exposure.

[b]A significant linear exposure concentration trend for increased incidence and or severity of alveolar/ductal hyperplasia of the mammary glands of male rats.

[c]A significant linear exposure concentration trend was reported for decreased live litter size in the F_1, F_2, and F_3, generation. No individual dose was significantly different from controls in the F_1 or F_3 generation.

From NCTR *(637)*.

Histopathological observations in adult males exposed during gestation and lactation included increased mammary gland hyperplasia in F_0 males of the 500 ppm group and F_1 and F_2 males of the 100 and 500 ppm groups. A significant linear exposure concentration trend for increased incidence and severity of alveolar/ductal hyperplasia was also reported in the F_1, F_2, and F_3 generations. Renal lesions were also observed in F_1 and F_2 males of the mid- and high-dose groups.

For body weight effects, only the most relevant effects (e.g., body weights including the time of pregnancy and terminal body weights) are summarized in **Table 121**. Body weights prior to and during gestation (≤13 weeks of age) were lower than controls in F_1 females exposed to ≥100 ppm and F_0 and F_2 females exposed to 500 ppm. Total body weight gain prior to delivery and terminal body weights of females were reduced in F_0, F_1, and F_2 animals of the 500 ppm group. Body weights of untreated F_4 females were slightly lower (<10%) but significantly different from controls during some periods before (8–11 weeks of age) and following (16–19 weeks of age) delivery of litters. Postweaning body weights in males were lower compared to controls in F_1 males exposed to ≥100 ppm during most time points in the post-weaning period. Total body weight gain throughout the study was decreased in F_1 males exposed to ≥100 ppm and F_3 males of the 500 ppm group; terminal body weight was significantly lower in F_1 males of the 500 ppm group. Significant and dose-related decreases in pup weight at birth were only observed in the F_5 generation, which received no genistein exposure. Genistein treatment decreased body weights of pups during the lactation period. Lower body weights were observed in males and females from the 500 ppm group on PND 21 in the F_1, F_2, F_3, and F_4 generations. Lower body weight was also reported for pups of the 500 ppm group on PND 14 including males of the F_1 and F_2 generations and females of the F_1 generation. Lower body weight in pups of the 100 ppm group was reported in F_1 males on PND 14 and PND 21, in F_3 males on PND 21, and in F_2 females on PND 14. **[The text of the results section stated that body weights of mid dose F_1 females were 12% lower on PND 14, but that statement could not be verified in the tables or figures of the report.]** Body weight was also reduced in male F_1 pups of the 5 ppm group. Body weight gain was reduced in F_1, F_3, and F_4 female pups of the 500 ppm group during the lactation period. Body weight gain of male pups during the lactation period was decreased in F_1 animals from the 100 and 500 ppm groups and in F_2, F_3, and F_4 animals from the 500 ppm group. Consistent reductions in feed consumption were observed in F_0, F_1, and F_4 females of the 500 ppm group. No consistent effects on water intake were observed.

In cases where organ weight effects were observed, the magnitude of effect was relatively small and there were no consistent effects across generations; therefore the biological significance was questioned by study authors. A small increase (8–9%) in testes weights in F_0 males of the 500 ppm group was the only effect on male reproductive organs; the study authors noted that the magnitude of effect was within variations observed in each dose group. Genistein had no detected effect on weights of female reproductive organs. Changes in weights of pituitary, thymus, and spleen in males and females were stated by study authors to be the only organ weight effects that differed by more than 10% from control values and were not related to body weight changes. A 17–18% increase in absolute and relative pituitary weight in the F_2 males of the 500 ppm group was the only organ weight effect that appeared to be dose-related.

Genistein exposure was associated with mammary hyperplasia and kidney effects in males. Incidence and severity of alveolar/ductal hyperplasia were increased in F_0 males of the 500 ppm group and F_1 and F_2 males of the 100 and 500 ppm groups. A significant linear exposure concentration trend for

increased incidence and severity of alveolar/ductal hyperplasia was also reported in the F_1, F_2, and F_3 generations. Kidney effects with increased incidence and severity (generation and doses at which effects occurred) included renal tubule mineralization (≥100 ppm in F_1 and F_2), renal cysts (500 ppm in F_1 and F_2), inflammation (500 ppm in F_1), and regeneration of tubules (500 ppm in F_1). All kidney lesions were rated minimal to mild. There were no other treatment-related histopathological findings.

Genistein treatment was associated with decreased live litter size and total number of pups born. Decreased live litter size and decreased total number of pups born was observed in the F_2 generation of the high-dose group. A significant linear exposure concentration trend for decreased live litter size and decreased total number of pups born in the F_1, F_2, and F_3 generations was also reported. Genistein treatment did not show a detectable effect on mating, fertility, or pregnancy indices in any generation. No genistein effect on duration of gestation was detected. There were no detected treatment-related effects on resorption sites in animals that did not become pregnant. Ovarian follicle counts were not observed to be affected by genistein treatment. In male rats, genistein had no detected effect on sperm parameters. Treatment-related effects observed in developing pups are outlined in (**Table 121**). A significant decrease in anogenital distance was observed in F_1 males of the 500 ppm group; the study authors stated that the decrease was within variances observed within treatment groups, including the control group. Reduced anogenital distances were also observed in F_1 and F_2 females of the 500 ppm group and mid-dose F_3 females. Again, study authors noted that the magnitude of effect was within variations noted in all dose groups. There were no detected significant or treatment-related effects observed for stillbirths, sex ratios, or postnatal survival.

Genistein treatment was associated with alterations to several markers of sexual development, particularly in female offspring. Age of vaginal opening was accelerated in F_1 and F_2 females and body weight at vaginal opening was reduced in F_1, F_2, and F_3 females of the 500 ppm group. A delay in testicular descent was only observed in F_3 males of the 500 ppm group. Genistein treatment had no effect on preputial separation. In the 2 weeks following vaginal opening, the number of abnormal estrous cycles, characterized by extended estrus or diestrus, was increased in F_1 rats of the 500 ppm group. Cycle lengths were increased in F_1 and F_2 females of the 500 ppm group. The increased number of cycles with abnormal diestrous or estrous stages in F_3 rats of the 500 ppm group was the only significant and dose-related effect reported in rats examined during the 10 days prior to necropsy. Examination of ovaries, vaginas, and uteri at necropsy did not show an effect of genistein on estrous cycle synchrony. The study authors concluded that there were no overt signs of toxicity, but following effects in this study were related to genistein exposure:

- Reduced body weight gains, accelerated vaginal opening, decreased anogenital distance, and altered estrous cyclicity in females continuously ingesting genistein at 500 ppm (~51 mg/kg bw/day);
- Some evidence of reduced litter size at 500 ppm in generations continuously exposed to genistein; and
- Hyperplasia of the male mammary glands and calcification of renal tubules at 100 and 500 ppm (~7 and 35 mg/kg bw/day); there were weaker effects on male mammary hyperplasia at 500 ppm in males exposed only as adults or exposed only *in utero* and through lactation.
- The only evidence for carryover of effects of genistein exposure into unexposed generations is decreased body weight gains in preweaning pups.

A separate report *(638)* described a substudy in which bone parameters were evaluated in F_1 and F_3 animals. Three exposure regimens were used (n=12–43/sex/dose): 1) F_1 weanling rats continued on the test diet to age 2 years (F_1C), 2) F_1 weanling rats continued on the test diet through PND 140 followed by control diet to age 2 years (F_1T140), and 3) F_3 weanling rats weaned to control diet and followed to age 2 years (F_3). At 2 years of age, blood was collected for measurement of alkaline phosphatase (ALP) and serum pyridinoline (PYD) and the rats were killed. The lumbar spine and the caudal vertebrae were evaluated by dual photon x-ray absorptiometry (DXA) for bone mineral density (BMD), bone mineral content (BMC), and bone area. The right femur was removed and the cross sectional area and marrow area were measured.

No effects of genistein on bone mineral density were detected in any group or between generations. The cross-sectional area of the femur was reduced in male rats treated continuously with genistein (F_1C) and also when treatment was discontinued at PND 140 (F_1T140). No group differences were seen in bone-related parameters of female rats treated until weaning (F_3). In F_1T140 females (exposed to genistein prenatally through PND 140), ALP was lower in the high-dose group. The BMC and bone area of the lumbar vertebrae were lower in F_1C females in the high-dose group, and the cross sectional area of the femur and serum PYD were also lower in this group compared to the other F_1C dose groups. Both male and female rats from the F_3 generation had larger bones than those from the F_1 generation, primarily due to the marked differences seen at the highest dose. Although there was a trend toward decreased body weight in females of the F_3 generation, lumbar vertebral BMC and area were both increased; total femoral cross-sectional area and marrow cavity area were also increased - specifically in the high dose. PYD was significantly higher in the F_3 female mid- and high-dose groups. In males, femoral cross-sectional area was significantly larger in the F_3 rats; cortical bone area was increased, but marrow cavity was unchanged. The authors concluded that the effects of genistein throughout the lifespan resulted in decreased bone size; although this decrease was attributed to the lower body weights observed, the authors still concluded that the decrease in bone size could reduce the force required to break the bone. The differences in bone size between the F_1 generation (continuous, lifetime exposure to genistein) and the F_3 generation (only exposed indirectly through the dam) suggest a developmental effect of exposure to a phytoestrogen-containing diet from previous generations.

Strengths/Weaknesses: Strengths include the use of dietary exposure with estimation of genistein intake, use of a soy-free diet, the determination of genistein levels in the diet given to the control group, evaluation of 3 dose-levels of genistein that were selected based on the results of a range-finding study, the large number of animals/group. A major strength is that the experimental protocol for a multigeneration reproductive study conducted under the auspices of the NCTR was thorough and undertaken using GLP guidelines. Because of the expense, logistics, and record-keeping requirements, few laboratories can efficiently complete these types of studies.

Utility (Adequacy) for CERHR Evaluation Process: This study has limited utility in the evaluation process, showing that the highest dose of genistein, 500 ppm (about 35 mg/kg bw/day), was associated with adverse effects on development. A limitation of the study for the evaluation of soy infant formula is that animals were treated during gestation, lactation, and after weaning, making it difficult to discern which effects may be due to treatment during lactation alone.

Laurenzana et al., 2002 ***(639)***, from the FDA and NIEHS, examined the effects of dietary genistein exposure during pregnancy and after birth on ERα expression and on hepatic enzymes involved

in testosterone metabolism. From GD 7 (plug date=GD 0) through weaning of offspring on PND 21 **[day of birth not defined]**, 5 Sprague Dawley rats/group were fed 5K96, a soy- and alfalfa-free diet, to which genistein (>99% purity) was added at 0, 25, 250, or 1250 ppm. Study authors estimated genistein doses at 2–200 mg/kg bw/day. Litters were culled to 4 males and 4 females on PND 4. Offspring were fed the genistein-containing diets **[assuming the same diet fed to dams]** from weaning through PND 50. Offspring were killed, and liver microsomes were obtained for *in vitro* analysis of 5α-reductase activity by incubation with testosterone, followed by thin-layer chromatography analysis of generated metabolites. Microsomal CYP2C and CYP3A protein levels were determined by Western blot. Cytosolic ERα was quantified using an immunohistochemical method and Western blot. Each analysis was conducted in 3 or 4 rats/sex/group. **[It was not stated how offspring from different litters were distributed among dose groups.]** Statistical analyses included 1-way ANOVA, Kruskal-Wallis test, and Dunnett's test.

Significant effects of genistein treatment on 5α-reductase-generated metabolites in males included ~2-fold increases in dihydrotestosterone (5α-androstan-17β-ol-3-one)/5α-androstane-3β (3-diol) and 7α-hydroxytestosterone metabolites at the 250 ppm dose **[~20 mg/kg bw/day based on authors' estimate for the 25 mg/kg bw/day group]**. A similar increase in dihydrotestosterone/3-diol metabolites was reported for females of the 250 ppm group **[data were not shown]**. Significant effects on testosterone metabolites generated through CYP2C11 included ~2-fold reductions in formation of 2α-hydroxy- and 16α-hydroxytestosterone in the 1250 ppm group. Significant effects on CYP expression in male rats included an approximately 75% increase in CYP3A protein at the 250 ppm dose but about a 50% decrease at the 1250 ppm dose. CYP2C protein expression was numerically increased in males of the 250 ppm dose and decreased at the 1250 ppm dose, but the effect did not attain statistical significance. No effects on CYP protein expression were observed in female rats **[data were not shown]**. ERα levels in liver cytosol were significantly increased in females and decreased in males of the 1250 ppm group. The study authors concluded that genistein can influence activity of testosterone metabolizing enzymes and ERα expression, but the effects cannot be directly associated with estrogenic activity.

Strengths/Weaknesses: Some of the rats in this study were from the Delclos *et al.*, 2001 *(239)* study. Strengths included use of soy- and alfalfa-free chow, use of 3 genistein doses (25, 250, 1250 mg/kg bw), feed was assayed to confirm levels of genistein, determination of feed consumption and genistein intake, and standardization of litters on PND 4. A weakness is that it was not clear if the litter used as the experimental unit.

Utility (Adequacy) for CERHR Evaluation Process: Endpoints examined are of limited utility alone in determining developmental effects, but may be helpful in interpreting results from other studies.

Masutomi et al., 2003 ***(640)***, supported in part by grants from the Japanese Ministry of Health, Labor, and Welfare, examined the effects of dietary genistein exposure during the perinatal period on male and female CD®(SD)IGS. Rats were fed CRF-1, a regular rodent diet containing soy, except from GD 3 (day of vaginal plug=GD 0) to PND 21 (day of delivery=PND 1) when the rats were given soy-free diet. Soy-free diet was prepared according to the NIH-07 formulation except that soy meal and oil were replaced with ground corn, wheat, and corn oil. Rats were randomly assigned to groups of 5 or 6 and given soy-free diet containing genistein (>97% purity) 0, 20, 200, or 1000 ppm **[mg/kg feed]**

from GD 15 to PND 10. Mean genistein intakes during gestation and lactation were estimated by study authors at 1.3–2.1, 13.7–23.0, and 66.6–113.1 mg/kg bw/day. The highest genistein dose was selected to produce weak systemic effects on the dam (e.g., decreased body weight gain) without affecting reproductive parameters. On PND 2, pup body weights and anogenital distance were measured. Litters were culled to 5–8 pups on PND 10. On PND 21, pups were weaned and given the CRF-1 diet. Five offspring/sex/group were necropsied on PND 21 for measurement of organ weights and volume of the sexually dimorphic nucleus of the pre-optic area (SDN-POA). Onset of puberty in males and females and estrous cyclicity in 8–11-week-old females was determined in 8 offspring/sex, which were ultimately killed and necropsied at 11 weeks of age. Females were killed during diestrus. Brain, adrenal, testis, ovary, uterus, pituitary, and ventral prostate weights were measured. Testes were fixed in Bouin fluid, and all organs except brain were examined histologically. Treatment groups from both time periods consisted of at least 1 pup/sex/litter. The litter was considered the statistical unit in evaluations conducted during the lactation period. For offspring data collected after weaning, individual animals were considered the statistical unit. Statistical analyses included Bartlett test, 1-way ANOVA, Dunnett's test, Kruskal-Wallis *H*-test, Dunnett's-type rank-sum test, Fisher's exact test, and Mann-Whitney *U*-test.

A tendency for decreased body weight gain during gestation was observed in dams of the high-dose group. No effects on feed intake during gestation or lactation were detected. Live litter sizes were not shown to be affected by genistein treatment. In offspring necropsied during the prepubertal period, no significant effects on body weight gain, anogenital distance, or brain, adrenal, testis, ovary, or uterus weights were detected. In controls and in all treatment groups, volume of SDN-POA was ~10 times higher in males than in females. In offspring necropsied in adulthood, there was a significant decrease in body weight gain in males of the high-dose group on PND 21–42. No effect of genistein treatment on onset of vaginal opening or preputial separation was detected. Body weights of high-dose males were significantly lower than controls at the time of preputial separation. All genistein-treated females had normal estrous cycles. All treated groups of males had significantly lower body weights than controls at necropsy. Significant organ weight changes in males included increased relative brain weight at the low dose, decreased absolute pituitary weight at the high dose, increased relative pituitary weight at the low dose, and increased relative adrenal weight at the mid and high dose. The study authors attributed organ weight effects to body weight changes. There were no histopathologic changes in those organs or other male organs, including testis and ventral prostate. Large atretic follicles were observed in ovaries of 2 females from the mid-dose group and 1 female of the high-dose group. However, no changes in mean numbers of secondary follicles or large atretic follicles per unit area were detected.

The study authors concluded that parameters related to sexual development were unaffected by genistein treatment. Reduced body weights of males after treatment ended was unexpected and of unknown biological significance.

Strengths/Weaknesses: Strengths of this study included use of soy-free chow with similar nutritional contents as soy-containing chow, use of 3 genistein dose levels, determination of feed consumption and genistein intake, and standardization of litter size on PND 10. Phytoestrogens were measured in chow, but no method and few data were presented. Estrous cycles were determined in adult females so all were sacrificed at the same stage of the cycle. Inclusion of histopathological assessment of

reproductive tract tissues provides added weight to the credibility of the lack of effect of treatments on these target tissues. Weaknesses of the study include cessation of genistein exposure on PND 10 and no assessment of reproductive capability of adult offspring. It is assumed that animals selected at weaning for further analysis were selected randomly.

Utility (Adequacy) for CERHR Evaluation Process: This study is of limited utility based on the route of administration, the thorough and careful collection of experimental data and exposure during relevant periods, but is limited by exposure duration. A limitation of the study for the evaluation of soy infant formula is that animals were treated during gestation and lactation, making it difficult to discern which effects may be due to treatment during lactation alone.

Masutomi et al., 2004 ***(641)***, supported by the Japanese Ministry of Health, Labor, and Welfare, reported immunohistochemistry studies performed on the pituitary glands obtained in the previous study *(640)*. Pituitaries from 5 animals per time point were evaluated at postnatal weeks 3 and 11 after maternal dietary genistein exposures of 0, 20, 200, or 1000 ppm **[mg/kg feed]** from GD 15 to PND 10. Immunohistochemistry was performed for LH, FSH, and prolactin. No effects of the treatments on the proportion of pituitary cells staining for any of these hormones were detected. The authors concluded that the exposure to genistein under the experimental conditions did not affect the developing hypothalamus-pituitary axis.

Strengths/Weaknesses: The chow and experimental design were the same as in studies by Takagi *et al.*, 2004 *(642)* and Masutomi *et al.*, 2003 *(640)*. These studies have similar strengths and weaknesses, including the use of multiple chemicals and doses.

Utility (Adequacy) for CERHR Evaluation Process: The study is of limited utility in the evaluation process. The data may be helpful in interpreting results from other studies. No effects of genistein on pituitary secretion of gonadotropins were noted in this study.

Takagi et al., 2004 ***(642)***, supported by the Japanese Ministry of Health, Labor, and Welfare, evaluated the effects of dietary genistein (to the dam during pregnancy and lactation) on ethinyl estradiol developmental toxicity in CD®(SD) IGS rats. Pregnant rats were obtained on GD 3 (plug=GD 0) and fed a soy-free diet. Beginning on GD 15, rats were divided into 5 treatment groups (n=6/group) and given: 1. soy-free diet; 2. dietary ethinyl estradiol 0.5 ppm alone; 3. dietary ethinyl estradiol 0.5 ppm with genistein (>97% pure) 100 ppm; 4. dietary ethinyl estradiol 0.5 ppm with genistein 1250 ppm; or 5 dietary genistein 1250 ppm alone. Dams were allowed to litter, and litters were standardized to 8 (4/sex where possible) on PND 3. Culled pups were used to provide trunk blood for determination of testosterone and 17β-estradiol. Treatments were stopped on PND 11, and all dams were switched to a standard laboratory chow derived in part from soybeans. According to the supplier, the genistein content was 102 mg/kg feed **[ppm]**, and the daidzein content was 87 mg/kg feed. Pups were weaned to this standard chow on PND 22 and housed with same-sex littermates up to 4/cage. Pups were observed for onset of puberty (preputial separation or vaginal opening), and during postnatal weeks 8–11 and 17–20, estrous cyclicity was monitored in 12 females/group (usually 2/litter). At least 8 offspring/group were killed during postnatal week 11 for evaluation of weight and histopathology of pituitary, adrenal, mammary gland, ovary, uterus, vagina, testis, epididymis, and ventral prostate. Males were killed on the first day of postnatal week 11, and females were killed on the next diestrus after the first

day of postnatal week 11, or on the first day if they entered postnatal week 11 in persistent estrus. An additional 8–13 females and an unspecified number of males were killed for similar evaluations at postnatal week 20. Comparisons were made by ANOVA with post-hoc Dunnett's test or by Kruskal-Wallis *H* test with post-hoc Dunnett's rank-sum test. Proportions were compared with Fisher's exact test and the severity of pathologic lesions with Mann-Whitney *U* test **[histologic change scored as –, ±, +, ++, or +++]**.

No influence of the coadministration of genistein was detected on the effects of ethinyl estradiol. **[Only the results of genistein alone in the diet will be given here.]** Genistein 1250 ppm in the diet had no detected effect on dam feed consumption or body weight. Calculated mean ± SD genistein intake was 96.1 ± 8.3 mg/kg bw/day on GD 15–20 and 196.5 ± 12.7 mg/kg bw/day on PND 3–11. Litter size was significantly decreased to 12.2 ± 1.33 in the genistein group compared to a control value of 14.1 ± 1.17. There were no observed genistein-associated alterations in pup body weight on PND 3, pup body weight gain during the lactation period, or pup survival to weaning. No significant genistein-associated alterations in serum testosterone or 17β-estradiol on PND 3 were detected. **[The authors described 17β-estradiol in males in the genistein group as "slightly increased without statistical significance." The concentrations estimated from a graph were about 220 ± 80 pg/ml in the genistein-exposed group and 80 ± 20 pg/ml in the control group. Errors were not specified in the graph but were SD elsewhere in the paper. The number of animals in each group was not specified except as "5 blood samples/group" in the Methods.]**

Age at puberty was said not to have been altered by genistein in either sex, although weight at preputial separation was greater in genistein-exposed males than in the control group (205.4 ± 17.6 g compared to 187.6 ± 13.5 g). **[Age at preputial separation was 41.0 ± 2.0 days in the genistein exposed group compared to 39.4 ± 1.3 days in the control group, *P*=0.0005, Student *t* test by CERHR using the number of offspring indicated in the data table (22 control, 23 genistein). Using n=6 litters, *P*=0.08 for a comparison of age at preputial separation.]** Monitoring of estrous cycles during postnatal weeks 8–11 showed prolonged diestrus in 7/12 genistein-exposed animals compared to 2/12 control animals. At postnatal weeks 17–20, the genistein-exposed group included 6/11 females with abnormal estrous cycles (2 with prolonged estrus and 4 with prolonged diestrus) compared to 1/12 animals in the control group with prolonged estrus. At both time points, the proportion of animals with abnormal estrous cycles was statistically increased in the genistein-exposed group. There were no histologic alterations in any male organs and no alterations in body or organ weights in either sex at either termination time. There was an increased incidence of endometrial and mammary hyperplasia in females exposed to genistein when evaluated at 11 weeks of age; mammary hyperplasia was also increased at 20 weeks of age. The study authors indicated that glandular hyperplasia and mucinous changes in the vaginal epithelium occurred in those animals showing prolonged diestrus, and in **20week**-old animals, cystically enlarged atretic ovarian follicles were seen in animals with prolonged estrus. The authors concluded that "[the effect of genistein] at 1250 ppm during GD 15–PND 11 is irreversible to the female endocrine/reproductive system even by maternal exposure, despite the effects bring rather weak as compared with those of [ethinyl estradiol]."

Strengths/Weaknesses: Strengths of this study include use of soy-free chow, measurement of 17β-estradiol, estrone, and phytoestrogen levels in chow, determination of feed consumption and genistein intake, standardization of litters to 8 pups on PND 3, and use of the litter as the experimental

unit. A weakness of the study was use of only 1 genistein dose level (1250 mg/kg); all other doses were administered in combination with ethinyl estradiol. Other weaknesses are that treatment was stopped on PND 11 and reproductive capability of animals was not examined.

Utility (Adequacy) for CERHR Evaluation Process: This study is of limited utility due to use of a single genistein dose level. However, the study aim was to evaluate the modifying influence of genistein on estradiol effects, thus it is not without utility.

Tousen et al., 2006 ***(643)***, supported by Health Sciences Research Grants from the Ministry of Health and Welfare, Japan, examined the effects of dietary genistein on dams and their offspring. Female Sprague-Dawley rats were received from CLEA (Tokyo, Japan) on gestation day 5. The rats were randomly assigned to experimental groups; feed intake of rats was adjusted to the mean feed intake of the control group; pregnant and lactating animals were housed on paper chip bedding. Pregnant rats were fed diet mixtures **[first day on diet was not clear]** containing genistein (94.85% pure) at dose levels of 0 and 0.5 g/kg; diets were prepared based on the modified AIN-93G diet except that soybean oil was replaced by corn oil. Two experiments were performed, Experiment #1 (n=16 rats) examined the effect of genistein exposure on dams during pregnancy and/or lactation; Experiment #2 (n=12 rats) examined the effects of genistein exposure of pregnant and nursing dams on their offspring in the growing period. *Experiment #1*: Pregnant rats were divided into 4 treatment groups (n=4 rats/group): the group fed the control diet, the group fed a genistein diet (0.5 g genistein /kg diet) during both pregnancy and lactation (PL-G), the group fed the genistein diet during pregnancy only (P-G), and the group fed the genistein diet during lactation only (L-G); all dams were placed on control diet after postnatal day 13 (PND 13). Maternal body weights and feed intake were recorded daily. On PND 1 (day of birth) the litter size, sex ratio and pup weights were recorded and the litter size was adjusted to 6 males and 6 females per litter. Blood was collected from all dams and 2 pups/sex/litter on PND 22 and pup organ weights were recorded. *Experiment #2*: Pregnant rats were assigned to two groups of 6 rats each: the group fed the control diet and the group fed a genistein diet (0.5 g genistein /kg diet) during pregnancy and until PND 13 (PL-G). From PND 22, to PND 75 F_1 rats were weighed weekly. Anogenital distance (AGD) was measured on PND 5, 15 and 22 as a measure of hormone imbalance. One male and 1 female pup/litter were killed on PND 5, 15, 22, 49, and 75 (2/sex/litter) and reproductive organ weights were recorded. Dams were killed on PND 22 and serum triiodothyronine (T3) and thyroxine (T4) were measured. *Both Experiments*: one-way ANOVA was used to determine the difference between treatments followed by Duncan's multiple range test. Regression analysis was used to examine the relationship between litter weight and litter size, and AGD and pup weight. Time-dependent changes were analyzed by repeated measures analysis of variance adjusted with degrees of freedom, following Huynh and Feldt. Significance was assigned at $P<0.05$.

Ingestion of genistein in the diet throughout pregnancy produced no significant differences in pregnancy outcomes, dam body weights, pregnancy or delivery. Pup body weights were not altered by exposure to genistein during pregnancy or lactation. There was no consistent affect on pup organ weights. Anogenital distance tended to be greater for males in all three genistein-treated groups on PND 15 and PND 22. Maternal circulating T3 levels tended to be lower in the P-G, L-G and PL-G groups; T4 levels tended to be lower in PL-G and L-G groups. There was no effect of maternal genistein exposure on pup reproductive organ weights.

Authors' conclusion: The exposure to genistein from the diet during pregnancy and lactation did not appear to induce serious influences on the offspring from birth to the post-weaning period. Maternal consumption of isoflavones did not have adverse effects on dams, fetuses, infants, suckling pups or offspring.

Strengths/Weaknesses: Strengths of the study include exposure to genistein via the diet, use of soy-free diet, information on compositions of diets were provided, determination of feed consumption and genistein intake, and exposure during critical periods of development. The design of experiment 1 was useful for examining the effects dietary exposure to genistein during pregnancy and/or lactation on development prior to weaning. Experiment 2 was designed to assess the effects of exposure during pregnancy and lactation on postnatal development through to adulthood. A weakness of the study included the use of only 1 genistein dose level (500 mg/kg genistein in the diet) and it is not clear as to why this dose was selected and is a high dose. Other weaknesses include the low numbers of animals evaluated (n=4-6 dams/group; 8 pups/sex/group), lack of mention that the authors verified the stability of the genistein in the diet, it is not clear if the litter was used as the experimental unit in the data analyses, and it is not clear if pup bodyweight was used as a covariate in the AGD analyses. Food intake was measured according to the methods but no data are reported, these data are needed to estimate the actual dose of genistein received. There was no measurement of circulating genistein level. In experiment 2 which evaluated effects on postnatal development there was no assessment of effects on vaginal opening or preputial separation, estrous cyclicity, tissue histomorphology, or fertility.

Utility (Adequacy) for CERHR Evaluation Process: The study has limited utility for the present evaluation since only one dose level was evaluated, and the number of animals and endpoints assessed were limited. The data may be useful for corroborating the results of other studies.

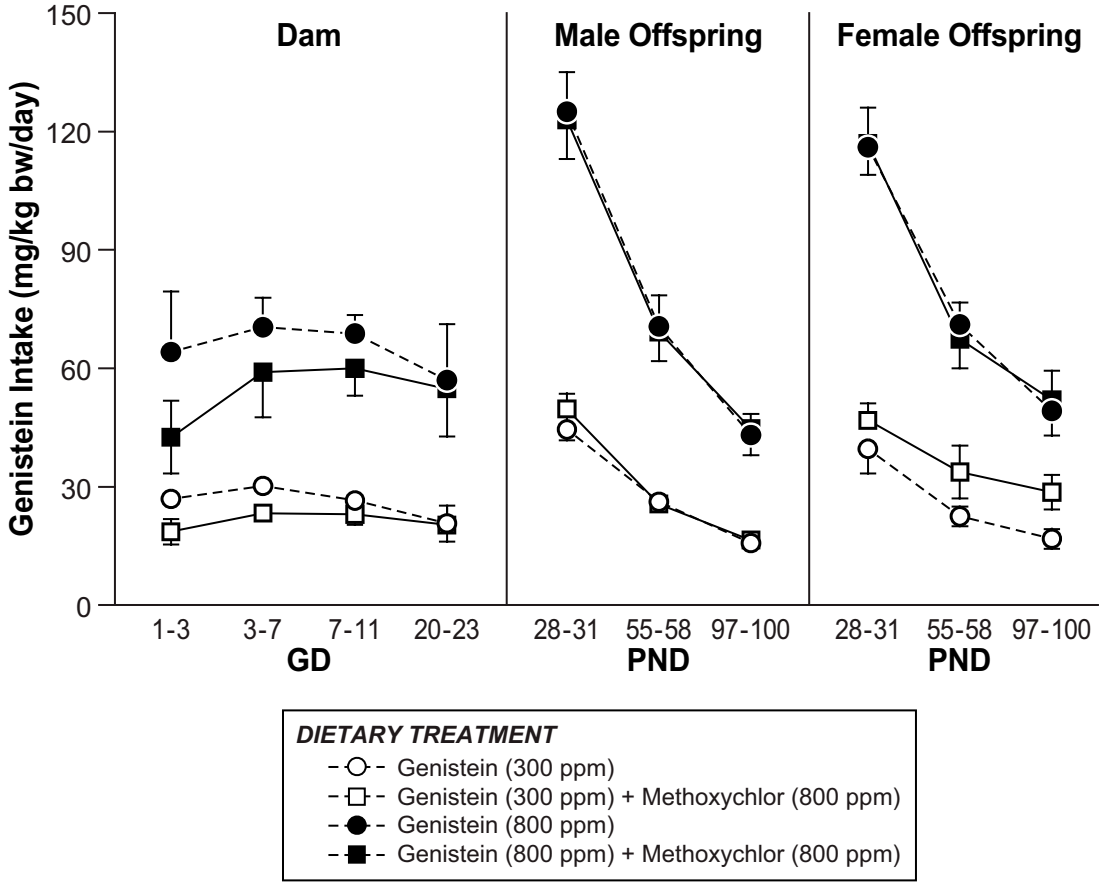
You et al., 2001 ***(633)***, from CIIT, evaluated the developmental effects of dietary genistein during pregnancy and after birth, alone and in combination with methoxychlor, a pesticide with an estrogenic metabolite (2,2-bis-(*p*-hydroxyphenyl)-1,1,1-trichloroethane [HPTE]). HPTE has ERα agonist activity and ERβ antagonist activity. Timed-mated Sprague Dawley rats were obtained on GD 0 (the day sperm were found in the vaginal smear). Animals were randomized by weight to 1 of 6 groups (8 animals per group). A control group was given untreated feed (a soy- and alfalfa-free diet). Treated animals were given the same feed with the addition of genistein (>98% pure), methoxychlor (~95% pure), or both. The 5 diet combinations were: 1. methoxychlor 800 ppm; 2. genistein 300 ppm; 3. genistein 800 ppm; 4. genistein 300 ppm + methoxychlor 800 ppm; and 5. genistein 800 ppm + methoxychlor 800 ppm. The 300 ppm dose of genistein was selected to approximate the amount of genistein in the NIH-07 rodent diet. The 800 ppm doses of genistein and methoxychlor were both based on previous studies showing endocrine effects at these exposure levels.

Dams were maintained on their assigned diets during pregnancy and lactation. Offspring were housed with their dams until weaning on PND 21. **[No statement was made about culling.]** On PND 22, one pup/sex/litter was killed and brain, liver, testis, ventral prostate, and uterus were dissected, weighed, and fixed in neutral buffered formalin for histologic evaluation. Dams were killed at this time and uteri evaluated for implantation sites. Retained offspring were housed 4 to a cage by litter and sex and fed with their dam's assigned diet until PND 90. Animals were observed for vaginal opening (from PND 25) and preputial separation (from PND 35). Daily vaginal smears were taken for 2 weeks

following vaginal opening to characterize the estrous cycle. On about PND 55, housing was changed to 2/cage by sex and litter. On PND 64–65, one male and one female from each litter were tested for spontaneous locomotor activity using a photobeam activity sensor system during the light phase of the photoperiod. On PND 64, animals were evaluated for 60 minutes following an ip dose of saline, and on PND 65, the same animals were evaluated after an ip dose of amphetamine. On about PND 110, three males (when possible) and 1 female offspring per litter were killed and organ weights obtained. On PND 120, estrous cyclicity was assessed for 3 weeks with daily vaginal smears in 2 females/litter. One of the 2 females/litter had been switched on PND 90 to the soy- and alfalfa-free diet without added genistein or methoxychlor. Statistical comparisons were made with 2-way ANOVA (genistein and methoxychlor as treatment factors). Body weight was used as a covariate when organ weights were analyzed. When observations were repeated over time, a repeated-measures ANOVA was used. When endpoints were assessed in more than 1 pup/sex/litter, a nested model was used to account for possible litter effects.

Offspring were weighed at birth, weekly during lactation, and on about PND 30, 55, and 100. Intakes of genistein and methoxychlor were estimated based on these weights and feed consumption, which was assessed by cage over a 3–4-day interval at various time periods. Feed intake was noted to be reduced by both genistein and methoxychlor. Estimated genistein intakes are given in **Figure 5**. Pregnancy

Figure 5. Estimated Genistein Intake in the Dietary Treatment Study



From You et al., 2002 *(633)*

exposures of the dam varied the least among groups given the same genistein feed concentration, ranging from ~19 to 30 mg/kg bw/day at 300 ppm genistein and from 42 to 64 mg/kg bw/day at 800 ppm genistein. Among offspring, genistein intake on a weight basis was greatest among prepubertal animals and decreased with age. The relatively high intake among prepubertal animals was attributed by the authors to a higher ratio of feed intake to body weight at this life stage than at older ages. There was little effect of methoxychlor on weight-adjusted genistein intake, which was attributed by the authors to a commensurate reduction in feed intake and body weight in animals exposed to methoxychlor. Estimated methoxychlor intake was 42–64 mg/kg bw/day in pregnant dams, 44–132 mg/kg bw/day among male offspring, and 52–120 mg/kg bw/day among female offspring. The presence of genistein in the diet did not affect methoxychlor intake except among prepubertal males. Lactation exposures were not estimated.

None of the treatments were shown to affect the number of implantation sites, embryo loss, or sex ratio. Genistein "marginally" increased litter size (P=0.051). The mean ± SD litter size in controls was 11.0 ± 1.6. Mean litter size in the group given 300 ppm genistein was 11.6 ± 1.8, and in the group given 800 ppm genistein, the mean litter size was 12.9 ± 1.8. **[N=8 litters/dose group. Test for linear trend performed by CERHR gave P=0.04 for these data; BMD_{10}[13]=502 ppm, $BMDL_{10}$=252 ppm, $BMD_{1\ SD}$=700 ppm, and $BMDL_{1\ SD}$=392 ppm.]** The body weight of male newborns was not shown to be affected by either genistein or methoxychlor treatment, although there was a significant interaction between the two treatments. The birth weight of female offspring was reduced by both treatments and by the interaction between the treatments. The mean ± SD birth weight of control females was 7.09 ± 0.34 g. In the group exposed to genistein 300 ppm, female birth weight was 7.06 ± 0.63, and in the group exposed to genistein 800 ppm, female birth weight was 6.51 ± 0.35 **[n=8 litters/dose group; BMD_{10}=812 ppm, $BMDL_{10}$=765 ppm, $BMD_{1\ SD}$=751 ppm, and $BMDL_{1\ SD}$=378 ppm]**. No effect of treatment on anogenital distance on PND 1 was detected. Treatment was said to have affected dam body weight at the end of the lactation period **[data including the direction of the body weight change were not given]**.

Offspring body weight on PND 22 **[PND 21 is indicated in a data table]** was decreased about 15% in males and 16% in females in the 800 ppm genistein exposure group. **[For males, BMD_{10}=779 ppm, $BMDL_{10}$=382 ppm, $BMD_{1\ SD}$=791 ppm, and $BMDL_{1\ SD}$=415 ppm; for females, BMD_{10}=595 ppm, $BMDL_{10}$=340 ppm, $BMD_{1\ SD}$=598 ppm, and $BMDL_{1\ SD}$=323 ppm.]** Genistein exposure did not affect PND 21 liver, brain, ventral prostate, testis, or uterine weights. Methoxychlor treatment resulted in a 3-fold increase in uterine weight. Genistein at 800 ppm delayed preputial separation when body weight was used as a covariate. **[The magnitude of the delay could not be estimated from the information provided.]** There was an interaction between methoxychlor and genistein in delaying preputial separation. **[Genistein added about 0.5 day of delay, estimated from a figure, and 1.3 days of delay according to the mean age of preputial separation given in the text.]** Vaginal opening was accelerated by genistein at both exposure levels. The average day of vaginal opening in the control females was PND 34; 300 ppm genistein advanced mean day of vaginal opening to PND 32, and 800 ppm genistein advanced mean day of vaginal opening to PND 28. A possible interaction between methoxychlor and genistein in vaginal opening could not be evaluated due to missing data.

[13] See the footnote to Table 95 for an explanation of the use of BMD in this report.

Offspring body weight on PND 110 **[PND 100 is indicated in the data table in the paper]** was reduced 10% in males and 8% in females by exposure to 800 ppm genistein. **[For males, BMD_{10}=812 ppm, $BMDL_{10}$=547 ppm, $BMD_{1\ SD}$=689 ppm, and $BMDL_{1\ SD}$=364 ppm; for females, BMD_{10}=802 ppm, $BMDL_{10}$=630 ppm, $BMD_{1\ SD}$=794 ppm, and $BMDL_{1\ SD}$=544 ppm.]** No treatment-related effects of genistein on weights of the ventral prostate, testis, epididymis, liver, brain, adrenal, uterus, or ovary were detected. Pituitary weight was increased 30% in male offspring exposed to 800 ppm genistein. No treatment effect on female pituitary weight was detected.

Genistein-related alteration in the estrous cycle during the 2 weeks following vaginal opening was not detected; however, in adult females, the time spent in estrus was increased. **[According to the text, the increase was seen in the 300 and 800 ppm groups; however, the bar graph showing the data clearly does not illustrate an effect at 300 ppm. The height of the bar indicating time spent in estrus is lower for the 300 ppm group than for the control group.]** Withdrawal of genistein treatment for a month prior to estrous cycle evaluation did not prevent the increased time spent in estrus, leading the authors to suggest that the alteration was not reversible. Histologic examination of male and female tissues showed no genistein-related changes; the only alterations noted were in the ovaries of methoxychlor-exposed animals, including animals exposed to methoxychlor + genistein. There were no effects of either genistein or methoxychlor, alone or in combination, on motor activity.

The authors noted that genistein is often identified by *in vitro* studies as a more potent estrogen than methoxychlor; however, in this *in vivo* study, methoxychlor appeared more estrogenic than genistein. Differences in kinetics were mentioned as a possible explanation for the differences in activities, but the authors also concluded, ". . . factors other than reactivity with sex hormone receptors may be responsible for some of the biological effects of these compounds."

Strengths/Weaknesses: Strengths of the study include exposure to genistein via the diet use of soy- and alfalfa-free chow, verification of uniform genistein blending in chow, determination of feed consumption and genistein intake, exposure during critical periods of development, and use of the litter as the experimental unit. The cross-over design was useful for examining reversibility of effects on estrous cyclicity (PND 90). Weaknesses of the study included the use of only 2 genistein dose levels (300, 800 mg/kg bw), fairly small numbers of animals/group (n=8), lack of examination of reproductive function, lack of mention that the authors verified the stability of the test materials in diet, measurement of pup body weights on approximate PNDs rather than exact PNDs, and fixation of testes in formalin, which is not the best fixative for histological examination of this tissue for staging of the spermatogenic cycle. Genistein 800 ppm decreased maternal feed consumption during gestation and lactation, which could have impacted other results (e.g., newborn female body weights). The authors' statement that the genistein-exposed female offspring spent an increased amount of time in estrus relative to controls is not verifiable from the study figure for the 300 ppm group. Sample sizes were insufficient for motor activity measurements as noted by the high coefficient of variation. Different potencies were exhibited *in vivo* (methoxychlor > genistein) than *in vitro* in the transcriptional activation assays for estrogenic activity (genistein > methoxychlor). Furthermore, genistein did not potentiate the effects of methoxychlor *in vitro* (androgen receptor transcriptional activation assay), but appeared to augment methoxychlor effects *in vivo* by extending the methoxychlor-induced delay in preputial separation. The high dose of methoxychlor was not realistic; consequently, the data may not reflect the interactions of these agents at low dose levels.

Utility (Adequacy) for CERHR Evaluation Process: This study is of limited utility based on use of only 2 dose levels and low number of animals evaluated. However, the data may be helpful in corroborating data from other reports.

3.3.2 Mammary Gland Development and Carcinogenesis

The effects of genistein on mammary gland development and/or carcinogenesis were studied in rodents exposed during prenatal or postnatal development. The studies are presented in order of mouse before rat and then alphabetically by first author.

3.3.2.1 Mice

Fielden et al., 2002 ***(645)***, in a study funded by the EPA, examined the effects of gestational and lactational oral exposure to genistein during gestation and lactation in mice on mammary gland development. C57BL/6 female mice were mated with DBA/2 male mice to produce B6D2F_1 offspring. The C57BL/6 mice were fed AIN-76A, a feed with undetectable levels of isoflavones, throughout pregnancy and lactation. Mice (a minimum of 9/group) were gavaged with genistein (98% purity) in corn oil 0, 0.1, 0.5, 2.5, or 10 mg/kg bw/day on GD 12 through PND 20, excluding the day of parturition (PND 0). The lower two doses represented human dietary exposures, while the highest two doses were selected to replicate potentially higher exposures resulting from supplement intake. Pups were weaned on PND 21. Litter size and weight were evaluated and anogenital distance was measured on PND 7 and 21. Mammary gland development was examined in females from 5–9 litters per group on PND 49. **[It was not clear if all females from each litter were examined.]** The selection of the time point for mammary gland evaluation was based upon the results of a preliminary study to assess mammary gland development in untreated mice. Effects were compared to those in mice exposed to diethylstilbestrol 0.1–10 μg/kg bw in a separate experiment with similar design. The litter was considered the experimental unit in statistical analyses that included ANOVA, ANCOVA, Dunnett's method, Tukey method, and Kruskal-Wallis test.

No effects of genistein treatment on body weight or anogenital distance were detected. Genistein treatment had no detected effect on percent mammary growth, mammary length, number of terminal end buds (a measure of proliferation), or number of alveolar buds (a measure of differentiation). In contrast, treatment with diethylstilbestrol 10 μg/kg bw increased percent mammary growth. Diethylstilbestrol was also reported to decrease the number of terminal end buds, but the effect was only marginally significant. The study authors concluded that gestational and lactational exposure to genistein at levels equivalent to or higher than that encountered by populations eating soy-rich diets does not affect mammary morphology in pubertal female mice.

Strengths/Weaknesses: A strength of this study is that pregnant dams were treated orally with genistein at dose levels comparable to human exposure (0.1 and 0.5 mg/kg bw/day) and higher levels to simulate dietary supplements. The use of multiple dose levels allowed for an assessment of dose-response relationships. AIN-76 diet with undetectable levels of genistein, daidzein, and glycitein was used in these experiments. Genistein was >98% pure. The authors described extensive characterization work aimed at delineating baseline mammary gland development in the mouse strain used and determined the optimum time point for assessing mammary gland development. Multiple time points (3, 4, 5, 7 and 10 weeks of age) were assessed. The authors controlled for litter effect by using 5–9 animals in each age group, with all animals originating from different litters. For genistein experiments,

gavage doses were adjusted daily to dam body weights. Anogenital distance measurements were made by a single observer to limit inter-experimenter variability. The same mammary gland in each animal (fourth abdominal gland on the right side) was used for each assessment. All mammary whole mounts were examined blind to treatment group by 2 people and averaged. The litter was used as the experimental unit. Statistical analyses were appropriate. The authors identified and included covariate terms that influenced endpoint measurements to account for sources of variability. They also adjusted for multiple comparisons to protect the α level at 0.05. Genistein results were compared with diethylstilbestrol-induced effects on mouse mammary gland development (evaluated in a separate study, but reported here). It is a weakness that the diethylstilbestrol and genistein experiments were not run concurrently, which may have contributed to difficulty in interpreting alveolar bud development in 7-week-old control mice. Dam blood levels of genistein were not measured in this study. Neither lactational transfer of genistein nor pup blood genistein levels was measured, making it difficult to assess the exposure to pups during the lactational period. The authors did not discuss how the critical windows for mammary gland development compare between mice and humans.

This is a subgroup report of animals from a study designed for another purpose; all endpoints in this study are secondary endpoints to those in the original study. This is often seen as a flaw in study design (the original study was designed, and presumably powered, to study male reproductive development and sperm quality). The study was done on a mixed genetic background for the study (DBA/1:C57BL/6 cross); only F_1 mice appear to have been used (comparing data with other published studies may not be straightforward). The DES and genistein experiments were not done at the same time and are essentially historical comparisons; usually a highly problematic approach for comparative studies. Problematic here because of testing a hypothesis based on estrogenic effects of genistein where a concurrent DES controls is the required positive control. Not clear if all females from each litter were evaluated, leading to potential bias. There did not appear to be any cross-fostering and offspring remained with dams until weaning. Higher exposure(s) may not be relevant to human exposures. Variability in several key measures is very high, particularly for primary mammary gland measures at center of study. i.e., TEBs, ABs; may reflect their role as secondary endpoints in a male reproduction study design (but reported here as primary endpoints), these effects (or any lack thereof) may well be unreliable. Mammary gland development was assessed at a single time point; will have missed any earlier/later events if these occurred.

Utility (Adequacy) for CERHR Evaluation Process: This study is of no utility in the evaluation.

Hilakivi-Clarke et al., 1998 ***(646)***, supported by the American Cancer Society and the Public Health Service, evaluated the effect of prenatal exposure to genistein via injection on mammary gland development in mice. Pregnant outbred CD-1 mice were obtained on GD 7 and injected on GD 15–20 with genistein 20 µg/day. **[The days of treatment were indicated only in the abstract; injection route was not specified. Number of treated dams and dam weights were not given; assuming a dam weight of 25–30 g, this genistein dose is about 0.7–0.8 mg/kg bw/day. Neither plug day nor day of delivery was specified.]** Other groups of pregnant mice were treated with 20 ng estradiol benzoate, 2 µg zearalenone, 2 µg tamoxifen, or oil vehicle. Within 24 hours of birth, males were removed and litters were constituted of 2 or 3 female pups born to a given dam plus 6 or 7 female pups fostered in from other dams in the same treatment group **[final litter size not indicated but presumably 9]**. Fifteen to thirty offspring/dose group were examined for eyelid opening beginning on PND 12, and 14–32 offspring/dose group were weighed on PND 25, 35, and 46. Four or five pups/dose group/

time point were killed on PND 25, 35, or 46 for measurement of serum 17β-estradiol and evaluation of mammary gland morphology by dissecting microscope examination of carmine aluminum-stained whole mounts. Day of vaginal opening was assessed in 10–25 pups/dose group. At 2 months of age, 6 offspring/dose group were monitored by daily vaginal smear for estrous cycling. Statistical comparisons were performed using ANOVA with post-hoc Fisher's Least Significance Difference test or nonparametric tests for proportions. **[Litter of origin appears not to have been tracked or considered in the analysis in spite of the dam having been the treatment unit.]**

There were no detected effects of treatment on number of offspring born or PND 1 body weights **[presumably pup body weight; data were not shown]**. Genistein- and estradiol benzoate-exposed pups had significantly increased body weights on PND 25 compared to control pups. **[A difference of 4 g was estimated from a graph.]** Eye opening was accelerated in the estradiol benzoate-exposed group and delayed in the genistein-exposed group. Vaginal opening was accelerated in offspring exposed to estradiol benzoate, genistein, or tamoxifen. Serum 17β-estradiol measurements on PND 25 and 35 were not significantly altered in any treatment group **[mean ± SEM 17β-estradiol concentrations on PND 25 estimated from a graph were 30 ± 10 pg/ml in control offspring, and 60 ± 14 pg/ml in genistein-exposed offspring, n=4 or 5/group, $P \approx 0.1$, Student *t* test by CERHR]**. No genistein-associated difference in estrous cyclicity was reported compared to controls. **[The Expert Panel noted that all 6 control animals had 4–5 day cycles compared to 2/6 genistein-exposed animals, $P=0.06$, Fisher's exact test by CERHR.]** The epithelial area of the mammary glands from genistein- and estradiol benzoate-exposed offspring was larger than in the control group on PND 35 but not on PND 25 or 46. The density of terminal end buds in the mammary glands was increased in the genistein-exposed group on PND 35 and 46 and in estradiol benzoate-exposed offspring on PND 46. There was no difference in differentiation of breast tissue, assessed using the density of terminal end buds and lobuloalveolar units, between genistein-exposed and control offspring. The authors concluded, "Maternal exposure to genistein during pregnancy, at a dose comparable to that consumed by Oriental women, has profound effects on mammary gland of female mouse offspring." They further concluded that genistein effects were similar to those of estradiol benzoate.

Strengths/Weaknesses: The study examined several endpoints relevant to the goals of this program. For example, a strength of this study is that effects of genistein on mammary morphology were compared with effects observed in previous experiments with estradiol. The authors reported that the genistein dose level was physiologically relevant. Multiple time points were assessed. The fourth abdominal gland was used for mammary gland assessments. To account for sources of variability, statistical analyses included covariate terms that influenced endpoint measurements. When collecting blood for measurement of serum 17β-estradiol levels, estrous stage was controlled (blood was collected when animals were in estrus). Time (presumably age of animals) and treatment were used as variables (2-way ANOVA) for the analysis of mammary gland structures. A weakness of this study is injection route was not specified. The purity of genistein was not given, and dose solutions were not analyzed for concentration, stability, or homogeneity. The authors did not mention the use of phytoestrogen-free diet, suggesting the possibility of additional genistein exposure. Because the authors used only 1 dose level of genistein, dose-response relationships could not be evaluated. Maternal/fetal blood levels of genistein were not reported. There were no details on how dams were assigned to treatment groups or how many dams were treated. The days on which the animals were sperm-positive (GD 0 or 1) or delivered offspring (PND 0 or 1) were not specified. There was no indication that the authors controlled for litter effects. While they cross-fostered

pups into different litters (2–3 pups stayed with the biological mother), this cross-fostering would only control for environmental factors such as maternal caregiving. There was no evidence that the authors considered litter of origin when assigning pups to different endpoints. With 4–6 litters per group, the n value would be 4–6 for each endpoint. The authors do not specify what kind of oil was used as a vehicle. They did not state whether mammary whole mounts were examined blind to treatment group. Body weight was not measured and therefore was not included as a covariate when analyzing maturational landmarks (eye opening and vaginal opening). In some cases (e.g., serum 17β-estradiol measurements, estrous cycle evaluations), sample sizes were too small.

Utility (Adequacy) for CERHR Evaluation Process: The route of administration, use of a single concentration of genistein, failure to control for litter effects and lack of clarity on the use of phytoestrogen containing diet limit the value of this study. This study has limited utility for the evaluation.

Padilla-Banks et al., 2006 ***(647)***, supported by NIEHS, examined the effects of genistein (98% pure) on the mammary gland in mice following treatment via sc injection to neonates. Time pregnant CD-1 (crl: CD-1 [ICR] BR) mice were obtained from the breeding colony at the National Institute of Environmental Health Sciences (NIEHS) in Research Triangle Park and allowed to deliver. Pups from several litters were pooled together, separated into two groups (male or female), and then randomly standardized to 8 female pups per dam. There were six litters per treatment group. Commencing on PND 1 and continuing through day 5, three groups of neonatal CD-1 female mice (6 litters / 48 mice per group) were administered daily subcutaneous injections of genistein suspended in corn oil at dose levels of 0.5, 5 and 50 mg/kg bw/day (Gen-0.5, Gen-5 and Gen-50). The control group (6 litters / 48 mice per group) received corn oil only on the same regimen. **[No analysis of the dosing formulations was presented.]** Mice were fed a NIH-31 mouse diet that had been analyzed for estrogenic activity. Based on a previous pharmacokinetic study conducted by this lab, the authors state the highest dose was expected to produce circulating serum levels in neonatal mice of 6.8 μM **[1838 ng/ml]** - slightly higher than the circulating serum levels reported by Setchell *et al.*, 1997 for human infants drinking soy-based formulas of ~1–5 μM **[~270 – 1351 ng/ml]**. Whole mounts of abdominal mammary gland were prepared at 4, 5 and 6 weeks of age and at 9 months of age (n=4-8/group at each collection age). Whole-mount analysis included: number of terminal end buds > 100 μm (TEB), ductal elongation, number of branch points, and percent of area occupied by mammary gland structures in the fat pad. At 5 and 6 weeks, Western blot, serum hormone level analysis (estradiol and progesterone), estrogen receptor ERα and ERβ, real-time RT-PCR and immunohistochemistry samples (progesterone receptor (PR), ERα and ERβ were taken of the abdominal mammary gland. At 5 weeks, 6 weeks and 9 months, the reproductive tract and ovaries were saved and examined histologically (H&E) for estrous cycle and the presence of ovarian corpora lutea (CL). At 2 months, 8 mice per group were cohabited with proven control males, allowed to deliver their litters, and observed for lactation ability; mammary gland function was determined by pup growth and survival of the F_1 generation (litter weights on day 2 and day 10, survival on day 22). Analysis of the number of TEBs, ductal elongation, branching, density, body weights, percent weight gain of pups, percent pup survival, circulating levels of estradiol and progesterone, real-time RT-PCR, and area image analysis was performed using ANOVA followed by Dunnett's test; $P < 0.05$ was considered significant.

There was no effect of treatment with genistein on body weights at 4, 5 or 6 weeks. At weeks 5 and 6, the estrous cycle was not affected at Gen-0.5 or Gen-5; all mice in the Gen-50 treatment group

Table 122. Mammary Gland Morphogenesis after Neonatal Genistein Exposure (Padilla-Banks et al., 2006)

Parameter	*Before Puberty*	*Active Ductal Morphogenesis*	
	Age: 4 Weeks	*Age: 5 Weeks*	*Age: 6 Weeks*
Number of TEB > 100 µm	↔	↓Gen-50	↓Gen-5.0 ↓Gen-50
Ductal elongation	↔	↔	↑Gen-0.5
Ductal growth	↔	↓Gen-50	↓Gen-50
Number of branch points	↔	↓Gen-5.0 ↓Gen-50	↔
Average area	↔	↔	↔

Gen-0.5, Gen-5 and Gen-50 = Genistein dose levels of 0.5, 5 and 50 mg/kg bw/day.
↑,↓,↔ Increased, decreased or no difference compared to controls.
From Padilla-Banks et al., 2006 *(647)*.

were in estrus or metestrus at the time of collection. Examination of mammary gland whole mounts collected before puberty (at 4 weeks of age) revealed no morphological differences in development after Gen treatment; however, mice treated with Gen exhibited developmental effects at week 5 and week 6 (**Table 122**).

Measurements of hormone receptor levels (**Table 123**) showed no effect of Gen exposure for 4-week old mice. Five- and six-week old mice had increased levels of progesterone receptor protein and estrogen receptor-β mRNA in the groups exposed to Gen; conversely, ERα expression was decreased in the groups exposed to Gen.

Table 123. PR, ERα and ERβ Expression after Neonatal Genistein Treatment (Padilla-Banks et al., 2006)

Parameter	*Age: 4 Weeks (Before puberty)*	*Age: 5 Weeks*	*Age: 6 Weeks*
PR protein expression	↔	↑Gen-0.5 ↑Gen-5.0	↔
ERα immunostaining	↔	↑Gen-0.5 ↓Gen-5.0 ↓Gen-50	↓Gen-0.5 ↓Gen-0.5 ↓Gen-0.5
ERα mRNA*	↔	↓Gen-0.5 ↓Gen-5.0 ↓Gen-50	
ERβ immunostaining	↔	↔	↔
ERβ mRNA*	↔	↑Gen-0.5	

Gen-0.5, Gen-5 and Gen-50 = Genistein dose levels of 0.5, 5 and 50 mg/kg bw/day.
*When animals 5 and 6 weeks of age were combined.
↑,↓,↔ Increased, decreased or no difference compared to controls.
From Padilla-Banks et al., 2006 *(647)*.

At weeks 5 and 6, there were no significant differences in circulating levels of estradiol or progesterone between mice in similar stages of the estrous cycle. An apparent trend towards lower progesterone levels in the Gen-50 mice was attributed to the higher percentage of these animals in estrus compared to the other groups. When cohabited with proven males, five mice from each group were plug-positive; no live pups were delivered in the Gen-50 group. There was no difference in the average number of pups per litter, litter weights, calculated average pup weights, or percentage of pups surviving until weaning between the control group and the 0.5 and 5 mg/kg/day genistein-treated groups. At 9 months, 8 mice in the Gen-0.5 treatment group showed estrous cycling similar to the controls and had similar numbers of CL. Mammary glands of all 8 Gen-0.5-treated mice were similar to the controls independent of the estrous cycle. Two out of 8 mice in the Gen-5 treatment groups were in persistent estrus with excessive cornification and no CL; the mammary gland from all Gen-5 mice exhibited altered morphology including reduced lobular alveolar development (8/8), dilated ducts (4/8), and focal areas of dilated beaded ducts. All 8 mice in the Gen-50 treatment group had marked decreased branching and alveolar development; dilated beaded ducts were also present in this group.

Author's conclusions: Developmental exposure to genistein at environmentally relevant doses alters murine mammary gland morphogenesis during puberty despite the lack of obvious effects before puberty. Hormone receptor levels in the mammary gland are altered after neonatal genistein treatment and there are also long-term effects on the mammary gland, including ductal epithelial hyperplasia, in the higher does of genistein.

Strengths/Weaknesses: Use of concentrations of genistein that are representative of human exposure and the inclusion of multiple dose groups that allow for characterization of dose-response relationships are strengths of this paper. The sample size used for each treatment group was statistically robust. Evaluation of mammary glands at multiple time points is also a strength. Assessment of reproductive function in addition to mammary gland morphogenesis is a strong feature of this study. Inclusion of molecular endpoints was another strong point of this paper. Of note the authors included measures of mRNA and protein expression for both estrogen receptor subtypes and the progesterone receptor. Immunohistochemistry allowed for localization of steroid receptor expression patterns in the mammary gland. Weaknesses of this study include the route of genistein administration which is not considered to be relevant to human exposure. Study addresses a relevant timing of exposure. Genistein source and purity are provided. Pups were cross-fostered and body weights were recorded. Multiple dosing was incorporated into the experimental design, allowing for the analysis of dose effects. However, it is not clear that a genistein-free/vehicle control group was used. Mammary glands were collected at several time points, allowing an analysis of developmental modifications over time. The litter appears to have been considered in the analysis.

The mixed-protein sources of the NIH-31 cereal-based diet are potentially problematic, as the sources of macronutrients and the sources and concentrations of micronutrients may not be adequately controlled (unlike the semi-purified AIN-based diets). Variations in micronutrients may compromise the results. However, the diet was shown to be free of estrogenic activity. Subcutaneous route of administration is not relevant to human exposures. Serum hormone levels of E2 and Pg were measured but not genistein (historical data were used to predict serum levels).

Utility (Adequacy) for CERHR Evaluation Process: This paper has limited utility for the evaluation owing to the route of administration used. However, the inclusion of multiple dose groups which cover an exposure range that produces circulating levels representative of human exposure is valuable. Moreover, the inclusion of measures of mRNA and protein expression coupled with immunohistochemistry at different developmental time points in a statistically robust sample of animals provides mechanistic insight that is valuable to assessment of genistein effects on mammary gland development and differentiation.

3.3.2.2 Rats

Cabanes et al., 2004 ***(648)***, supported by the Komen Breast Cancer Foundation, the American Institute for Cancer Research, and the Cancer Prevention Foundation of America, examined mechanisms of breast cancer inhibition following prepubertal genistein exposure via sc injection in rats. Female Sprague Dawley rat pups were cross-fostered to dams on PND 2. **[The number of litters represented was not specified.]** On PND 8–20 **[day of birth not defined]**, the pups were sc injected with peanut oil (vehicle control), genistein **[purity not specified]** 50 µg/day, or 17β-estradiol 10 µg/day. Based on actual body weights of animals on the first and last days of treatment, the study authors estimated that the doses received were 1.25–3.3 mg/kg bw genistein and 0.25–0.67 mg/kg bw 17β-estradiol. **[The dams were fed Purina 5001 chow, but the feed given to pups following weaning was not specified.]** Rats were killed at 3 and 8 weeks of age (n=6 or 7/group/time period) to obtain mammary glands for morphology evaluation and mRNA and protein isolation. Mammary gland expression of BRCA1, a tumor-suppressor gene involved in DNA damage repair, was determined by RNase protection assay. ERα expression in mammary gland was determined using immunocytochemistry and Western blot methods. Rats treated with 17β-estradiol were evaluated at more time periods and for more parameters, but because genistein is the focus of this report, 17β-estradiol results are only discussed for parameters and ages for which genistein was also evaluated. Data were analyzed by 1- or 2-way ANOVA.

Tumorigenesis following treatment with dimethylbenzanthracene was examined in the rats treated with 17β-estradiol. Similar to results noted in a previous study with genistein treatment of immature rats *(323)*, 17β-estradiol treatment decreased the risk of developing dimethylbenzanthracene-induced tumors. There were no detected effects on mammary structures at 3 weeks of age. At 8 weeks of age, genistein treatment significantly reduced mammary epithelial density and terminal end bud numbers and increased lobuloalveolar structures; 17β-estradiol treatment significantly reduced terminal end bud numbers. Expression of BRCA1was significantly up-regulated in the genistein and 17β-estradiol groups at 3 and 8 weeks of age **[~1.5–1.75 fold increases in genistein compared to control group]**. Genistein treatment induced a significant increase in ERα protein expression in lobules at 8 weeks of age **[~1.5-fold increase compared to control group]**. Expression of ERα protein in ducts was significantly decreased in 8-week-old rats that received 17β-estradiol treatment. The study authors concluded that increased expression of BRCA1 may be a mechanism of reduced mammary cancer risk in rats following prepubertal genistein exposure.

Strengths/Weaknesses: A strength of this study is that the number of animals per condition was acceptable. The genistein dose was relevant to human exposure although the sc route was not. Results were compared with those of 17β-estradiol. Endpoints evaluated included general reproductive parameters (organ weight, vaginal opening, 17β-estradiol levels). The study examined potential molecular targets (e.g., BRCA1, ERβ) of genistein in mammary gland but not at all time points. A

weakness of the study is that only 1 dose level was tested. The study did not examine all endpoints (BRCA1, tumorigenesis, ERβ expression) in genistein-exposed rats. Because of data in other published reports, tumor incidence following genistein exposure should have been tested and data should have been presented for a few time points. Similarly, changes in mammary epithelial trees were not evaluated at 3 weeks, although previous studies showed that the response at that age differed from that of older rats. Although the abstract stated that both 17β-estradiol and genistein up-regulated BRCA1 expression at 3, 8, and 16 weeks, no data were shown for genistein at 16 weeks. Considering that 17β-estradiol and genistein were reported to have opposite effects on ERα expression, it is not possible to extrapolate that they would have the same effect on BRCA1 expression at all ages. Rats were maintained on Purina 5001 chow, which is an "open diet" with variable ingredients; thus, the proportion of genistein may have varied from batch to batch and could not be estimated.

Utility (Adequacy) for CERHR Evaluation Process: This study is of limited utility due to route of genistein administration, lack of dose-response information and missing critical time points for genistein-exposed rats. However, the study presents some additional mechanistic information to explain possible protective effects of prepubertal genistein exposure against breast tumor formation.

Fritz et al., 1998 ***(209)***, funded by NIH, explored the possible role of dietary genistein during early adulthood in protection from mammary tumors in rats. Seven-week-old female Sprague Dawley rats were treated with dietary genistein (98.5% pure, with 1.5% methanol) at 0, 25, or 250 mg/kg diet **[ppm; doses would likely be ~0, 2.2, and 22 mg/kg bw/day according to information presented in Fritz et al., 2002** ***(212)*****]**. The basal diet was AIN-76A, a phytoestrogen-free rodent feed. At 9 weeks of age, females were bred 2:1 with males that had been placed on the same diet as the females at the time of mating. Offspring were sexed at birth. Litters were standardized to 10 pups with 4–6 females. Offspring were weaned on PND 21 and given the untreated AIN-76A diet. On PND 50, female offspring were given dimethylbenzanthracene 80 mg/kg bw by gavage in order to induce mammary tumors. Animals were killed when palpable tumors reached 2.5 cm in diameter, when the animals became moribund, or on PND 200. Whole mounts of mammary glands were prepared from females on PND 21 and 50. **[The source and number of these animals were not specified.]** Mammary gland size and numbers of terminal end buds, terminal ducts, and lobules were determined. Uterine weights were obtained**.** Two hours before death, animals were injected with bromodeoxyuridine for labeling of proliferating cells in the mammary glands. **[The source and number of these animals were not specified, but they were at PND 21 and 50 and may have been the same animals used for the whole mounts.]** Serum genistein concentrations were measured in PND 21 offspring. **[The source and number of these animals were not specified.]** Serum testosterone concentrations were said to have been determined by RIA **[no results presented]**, and estrous phase was evaluated on PND 41–50. Total and free genistein levels were measured analytically in dam serum and milk at 7 days postpartum (free genistein in milk analyzed for high-dose dams only). Milk also was collected on PND 21, although these analytical results were not shown. Total and free genistein concentrations were measured in pup stomach milk (7-day-old pups only) and in pup serum and mammary glands at 7 and 21 days of age. The number of tumors per animal and time of tumor appearance were analyzed using a Poisson and Weibull distribution. ANOVA was used for other comparisons. **[Apparently none of the analyses considered litter of origin.]**

Genistein concentrations in mammary glands and milk are presented in **Section 2**. The number of litters produced by females in each of the treatment groups was expressed as follows: control diet 35/40; 25

ppm genistein 25/29; 250 ppm genistein 44/57. **[The Expert Panel assumes these data represent number of dams producing litters/number mated; there is no significant difference between these proportions.]** No significant differences were detected between groups in number of male or female offspring, anogenital distance, or time to testicular descent or vaginal opening. Among female offspring, there were no detected differences among treatment groups with respect to body weight, uterine weight, or mammary gland surface area at either PND 21 or 50, and no significant differences in time spent in each phase of the estrous cycle, number of primordial follicles, or number of corpora lutea in the ovaries. Histologic evaluation of the vagina, uterus, and ovaries showed no alterations on PND 50 or 100.

Genistein-exposed females developed fewer tumors per animal (control 8.8 ± 0.8 tumors/animal; genistein 25 ppm 7.1 ± 0.8 tumors/animal; genistein 250 ppm 4.4 ± 0.6 tumors/animal). **[The error was not defined; SEM was used elsewhere in the paper for other data. The number of animals or number of litters involved was not given.]** There was no detected alteration in latency to onset of tumor palpability. On PND 21 and 50, there were fewer terminal end buds in the group exposed to genistein 250 ppm. Type I lobules (defined as having 5–10 alveolar buds) were reduced in number by both genistein exposure levels on PND 50. There was no detected effect of genistein on numbers of type II lobules (10–20 alveolar buds) or on DNA labeling indices of mammary end buds or terminal ducts.

The authors concluded that neonatal exposure to genistein protected against mammary cancer in rats. Although they noted that the DNA labeling index was not altered by genistein, they calculated that multiplying the labeling index by the number of proliferating structures (e.g., end buds) showed a genistein-associated decrease in the total amount of cell proliferation in tissues at risk for carcinogenesis.

Strengths/Weaknesses: A strength of these experiments is that phytoestrogen-free AIN-76 diet was used. Genistein was 98.5% pure. According to data in another publication by this author *(212)*, exposure levels are relevant to human exposures, as are the oral route of exposure and exposure during the neonatal period. Mammary morphology was assessed at two time points (21 and 50 days of age). Histological examinations of tumors and estrous cycles were conducted blind to treatment group. Total and free genistein levels were measured analytically in dam serum and milk at 7 days postpartum. Total and free genistein concentrations also were measured in pup stomach milk (7-day-old pups only), and in pup serum and mammary glands 7 and 21 days after delivery. The use of multiple dose levels allowed for an assessment of dose-response relationships. Statistical analyses for tumor data were appropriate, although the influence of litter of origin was never tested for tumor data or other endpoints. A weakness of this study is that diets were not analyzed for concentration, stability, or homogeneity. There was no indication that the authors controlled for litter effects by selecting pups from different litters for each endpoint or controlling for litter of origin during data analyses. It was difficult to determine the sample size in many of the experiments. Serum testosterone data were not presented. Tumor incidence (number or proportion of animals developing tumors) was not given. The dimethylbenzanthracene dose level was relatively high (80 mg/kg bw); thus, this experiment was apparently designed to detect only decreases in tumor incidence. Blood genistein concentrations in neonatal rats were considerably lower than blood genistein concentrations reported for infants consuming soy formula.

Utility (Adequacy) for CERHR Evaluation Process: This study has limited utility, particularly the toxicokinetics data involving milk transfer and pup exposures. Moreover, use of concentrations of genistein that produce circulating levels representative of human exposure is valuable. The study is

less useful for the cancer endpoints because the dose of DMBA is high and will not detect increased tumorigenesis; thus, data and overall interpretations may be biased. DNA labeling index analysis is weak and probably unreliable.

Foster et al., 2004 ***(649)***, supported by the Canadian Chemical Producers association, Health Canada, and the Natural Sciences and Engineering Research Council, evaluated the effect of neonatal genistein exposure via sc injection in Sprague Dawley rats with antenatal exposure to a mixture of 17 different chlorinated compounds. The mixture was formulated to include organic and inorganic environmental chemicals for which there was evidence of human exposure in Canada. The compounds were present in amounts (on a mg/kg bw/day basis) representing "safe" exposure levels based on US or Canadian government regulations. Pregnant animals were gavaged with corn oil (n=9) or with the mixture (n=10) daily on GD 9–16 **[plug day not defined]**. On PND 2–8, half the pups in each group were given sc genistein 10 mg/kg bw/day **[assignment by litter not indicated]**. On PND 200, one female per litter were killed, and the first right thoracic mammary gland was dissected for histopathologic evaluation. **[There were 7 females evaluated from the group that was exposed to the mixture and to genistein, suggesting that more than one female/litter was used in this group.]** Histopathology findings were ranked from 0 (normal) to 4 (severe changes), with a decimal added to the integer to represent focal changes (0.25), locally diffuse changes (0.5), and diffuse changes (0.75). The maximum histologic abnormality, then, was represented by a rank score of 4.75. Comparisons were made using ANOVA with post-hoc Dunn test.

In the control group (corn oil during pregnancy, sc vehicle neonatally), there was one animal of the four examined with rare mild ductal hyperplasia. There were no detected histologic abnormalities in the group exposed during pregnancy to the mixture with neonatal vehicle administration. In the group exposed to corn oil during pregnancy and genistein in the neonatal period, mammary glands showed evidence of lactation with cystic ductal dilatation, atypical epithelial hyperplasia, and microcalcifications. *In situ* ductal carcinoma was identified in 2 of the 5 animals examined. In the group exposed to the mixture during pregnancy, these changes were more severe, with atypical hyperplasia in 6 of the 7 animals examined; however, there were no instances of carcinoma in this group. The authors concluded that low concentrations of environmental toxicants can interact with hormonally active agents postnatally to alter mammary gland structure. The authors contrasted their findings with those of Fielden *et al.*, 2002 *(645)* in which there was no adverse effect of *in utero* or lactational exposure of mice to genistein, and noted possible differences in route of administration (sc compared to oral). They also noted the discrepancy between their findings and those of Cotroneo *et al.*, 2002 *(650)*, who used the same rat strain and route of administration of genistein (at 500 mg/kg bw/day), but who did not find histologic changes suggesting an increase in mammary gland susceptibility to carcinogenesis. Cotroneo *et al.*, exposed animals to genistein on PND 16, 18, and 20 as opposed to PND 2–8 in the current study, and the current study authors concluded, "…our data suggest that both the dose and timing of exposure are critical factors in altering mammary-gland sensitivity to genistein-induced changes in mammary gland morphogenesis and potential tumorigenesis."

Strengths/Weaknesses: A strength of this study is that it included genistein exposure during the neonatal period. Histopathology was scored for both severity of changes and distribution. Statistical analyses were appropriate. The purity of genistein was not given, and dose solutions were not analyzed for concentration, stability, or homogeneity. The animals received standard laboratory rat chow (8604

Harlan Teklad, Madison, WI), which may have contributed additional genistein exposure. Because the authors used only 1 dose level of genistein (10 mg/kg bw/day), dose-response relationships could not be evaluated. The dose level was higher than human exposures (considered pharmacological, presumably because it may be in the range of some dietary supplements). Pup blood levels of genistein were not reported. There were no details as to how dams and pups were assigned to treatment groups. Sample sizes were relatively small (n=4–7 animals/group). Focal mild ductal epithelial hyperplasia was noted in 1/4 control animals. The authors reportedly controlled for litter effects by selecting 1 female from each litter for necropsy on PND 200; however, the n value of 7 was inconsistent with the 5 litters assigned to the mixture plus genistein treatment group, suggesting that the authors failed to control for litter effects when assigning animals to different endpoints and did not use the litter as the unit of analysis.

Utility (Adequacy) for CERHR Evaluation Process: This study is of limited utility in the evaluation process. Dosing and inadequate control for other nutrients render the study largely uninterpretable.

Hilakivi-Clarke et al., 1999 ***(651)***, supported by the American Cancer Society and the Public Health Service, evaluated the effect of prenatal genistein exposure (via sc injection to the dam) on susceptibility to dimethylbenzanthracene induction of mammary cancer in rats. In experiment 1, pregnant Sprague Dawley rats were obtained on GD 10 and treated with daily sc doses of genistein 20 μg (n=10), zearalenone 20 μg (n=11), or vehicle (n=9) on GD 15–20 **[plug day not specified; dam weight not given, but genistein dose was indicated as 0.1 mg/kg bw/day, implying a 200 g dam body weight]**. In experiment 2, dams were treated on GD 15–20 with sc genistein 0, 100, or 300 μg/day (stated to be 0.5 and 1.5 mg/kg bw/day). On PND 2, males were removed and female pups cross-fostered to produce litters of 10–12 pups. **[The Expert Panel questions whether pups were cross-fostered in the sense of pups being raised by dams in a treatment group other than that of their biological mother. In a previous publication from this laboratory *(646)*, the term "cross-fostering" was used to mean re-allocation of pups to dams within the same treatment group.]** In experiment 1, five offspring/group/time point were killed on PND 21 and 35 for estimation of ERα and ERβ protein in mammary glands using a ligand binding assay. In experiment 2, ER protein was estimated in 5 offspring each from the 0 and 300 μg genistein groups on PND 45. Protein kinase C was estimated in mammary tissue from 5 offspring/treatment group in experiment 1 using a commercial kit. In experiment 1, 45-day-old offspring (24/group) were treated with the mammary carcinogen dimethylbenzanthracene by mouth at 10 mg **[described as 40 mg/kg bw, implying 250 g body weight]**. The same dimethylbenzanthracene treatment was given in experiment 2 on PND 50 (18–27/group). Animals were evaluated weekly for number of animals with tumors, latency to the appearance of tumors, and number of tumors per animal. Animals were killed when their tumor burden reached 10% of their body weights or by 18 (experiment 1) or 20 (experiment 2) weeks after administration of dimethylbenzanthracene. **[Statistical methods were not explicitly discussed but appeared to be ANOVA with post-hoc Fisher's Least Significant Difference test. Litter of origin appears not to have been considered in the analyses.]**

Dams producing litters, dam weight gain, pregnancy length, pups/litter, and PND 2 pup weight were not found to be altered by treatment **[data shown only for experiment 1]**. There was no detectable effect of treatment on pup body weight in experiment 1. In experiment 2, pup body weights were ~9% lower than control in both genistein-exposed groups on PND 35 but not at earlier or later evaluations. Mammary ER protein content on PND 35 was nearly twice as high in pups born to dams treated with

genistein 20 μg/day compared to controls. On PND 45, ER protein content in mammary tissue was more than 5 times as high in pups born to dams treated with genistein 300 μg/day compared to controls. **[ER comparisons estimated from a graph; both comparisons were statistically significant according to the study authors.]** In experiment 1, there was no detected effect of treatment on protein kinase C activity on PND 21, but on PND 45, offspring born to dams treated with genistein 20 μg/day had a statistically significant 47% reduction in protein kinase C activity **[estimated from a graph]**. The incidence of mammary tumors after dimethylbenzanthracene treatment was significantly increased in offspring born to dams treated with genistein 20 or 300 μg/day but not 100 μg/day. There was no detected treatment effect in either experiment on latency to tumor development, number of tumors per animal, or number of tumors showing regular growth. The authors concluded that maternal exposure to genistein during pregnancy at doses in the range of human exposures increased susceptibility to carcinogen-induced mammary tumorigenesis.

Strengths/Weaknesses: A strength of this study is that female rat pups were cross-fostered on PND 2 to control for environmental factors such as maternal caregiving. Dose levels were selected to approximate the level of human exposure (0.1, 0.5, 1.5 mg/kg bw compared to reported human exposures of ~0.1 mg/kg bw in Asian populations), although the injection route is not relevant to human exposure. Multiple time points were assessed for mammary ER numbers, although the exposures were not the same at all time points. Appropriate controls were included in protein kinase C experiments. A dose of dimethylbenzanthracene was selected that allowed for detection of both decreases and increases in mammary tumors. The authors did not mention the use of genistein-free diet, suggesting the possibility of additional genistein exposure. The authors used only 1 dose level of genistein (20 μg) in Experiment 1, so dose-response relationships could not be evaluated. The authors estimated the 20 μg dose of genistein to be equivalent to 0.1 mg/kg bw/day (implied dam weight=200 g). There appeared to be an error, because it seems highly unlikely that female Sprague Dawley rats on GD 15–20 weighed as little as 200 g, particularly given that the animals dosed with dimethylbenzanthracene at 45 days of age were calculated to weigh 250 g. Maternal/fetal blood levels of genistein were not reported. The day on which the dams were sperm-positive was not identified (GD 0 or 1). There were no details on how dams were assigned to treatment groups, and there was no indication that the authors controlled for litter effects. While they cross-fostered pups into different litters (2–3 pups stayed with the biological mother), this cross-fostering would only control for environmental factors (see above). There was no evidence that the authors considered litter of origin when assigning pups to different endpoints. The ER assay measured total ER without specifying subtype (ERα or ERβ). The description of the statistical analyses was inadequate; tests were identified in cases where statistical significance was observed but not identified in cases where effects were not statistically significant. Body weights of female offspring were significantly lower at 35 days of age at both 100 and 300 μg genistein, which may have influenced some endpoints. There was a lack of consistency between doses of genistein used and time points at which data were collected. For example, reproductive endpoints (pregnancy rates, weight gain during pregnancy, numbers of pups/litter, etc) were examined only at 20 μg genistein, not at higher doses. ER protein levels were measured in the 20 μg group at 21 and 35 days of age and in 300 μg group at 45 days of age; thus, dose-response could not be assessed at any of those time points. A similar situation existed for protein kinase C activity. There was a discrepancy in the mammary tumor incidence between experiments 1 and 2. In experiment 1, 50% of control animals had tumors and 96% of animals given 20 μg genistein developed tumors by week 18. In experiment 2, only 17% of control animals developed tumors, compared with 27 and 44% of animals exposed

to 100 and 300 µg genistein, respectively. The authors mentioned that the difference in the control incidence was related to the age at which dimethylbenzanthracene was administered (45 days of age in experiment 1 compared to 50 days of age in experiment 2). It is not clear why dimethylbenzanthracene was administered at different ages, as this difference complicates the interpretation of the genistein results. Sample sizes were insufficient for some endpoints (e.g., only 3 animals developed tumors in the experiment 2 control group).

Utility (Adequacy) for CERHR Evaluation Process: The use of sc injection and the small sample size for some endpoints are limiting. The lack of clarity on the diet used is a problem. This study has limited utility for the evaluation.

Hilakivi-Clarke et al., 1999 ***(652)*****,** in a study supported by the American Cancer Society, the Lombardi Cancer Center, and the Public Health Service, examined the effects of a physiologic dose of genistein administered by sc injection on mammary tumorigenesis. Neonatal Sprague Dawley rats (n=30/group) were randomized to make litters of 10–12 females/dam. The rats were sc injected with 0 or 20 µg genistein **[purity not specified]** in a DMSO/peanut oil vehicle on PND 7, 10, 14, 17, and 20. Authors estimated doses at 2 mg/kg bw on PND 7 and 0.7 mg/kg bw on PND 20. On PND 45, rats were gavaged with ~50 mg/kg bw dimethylbenzanthracene to induce mammary tumors. Animals were examined regularly for up to 19 weeks following dimethylbenzanthracene dosing, at which time they were killed for an evaluation of mammary gland morphology (n=4 or 5/group) and the number of ER-binding sites in the 4th mammary gland (n=7/group). Data were analyzed by ANOVA, Fisher's Least Significant Difference, and chi-squared test.

No effect of genistein treatment on body weight gain was detected. The incidence of mammary tumors was 43% in the genistein group at week 18 compared to 57% in control group (not statistically significant). Tumor multiplicity was significantly reduced in the genistein group with a mean ± SEM of 1.1 ± 0.1 tumors/animal versus 1.8 ± 0.3 tumors/animal in controls. The percentage of proliferating tumors was also reduced in the genistein group (60%) compared to the control group (94%). Adenocarcinomas represented 100% of tumors in the control group and 40% of tumors in the genistein group. The remaining tumors in the genistein group were non-malignant. Genistein had no detected effect on tumor latency. Rats treated with genistein had greater lobular differentiation, significantly decreased terminal duct density **[about half that of controls]**, and significantly increased alveolar bud density **[~45% higher than controls]**. Genistein had no effect on ER protein levels in mammary gland. The study authors concluded that in rats, the risk of developing mammary tumors is reduced by a low dose genistein exposure prior to puberty.

Strengths/Weaknesses: A strength of this study is that rat pups were cross-fostered on PND 2, prior to genistein treatment. Dose levels were selected to approximate the level of human exposure in Asian populations. A dose of dimethylbenzanthracene was selected that allowed for detection of both decreases and increases in mammary tumors. Histological examinations were conducted independently by 2 pathologists who were blind to treatment group. The purity of genistein was not given, and dose solutions were not analyzed for concentration, stability, or homogeneity. The authors did not mention the use of genistein-free diet, suggesting the possibility of additional genistein exposure. The authors used only 1 dose level of genistein (20 µg), so dose-response relationships could not be evaluated. Genistein doses varied from 2 mg/kg bw on PND 7 to 0.7 mg/kg bw on PND 20. Maternal/fetal blood

levels of genistein were not reported. There were no details as to how dams were assigned to treatment groups, and there was no indication that the authors controlled for litter effects by considering litter of origin when assigning pups to different endpoints. The ER assay measured total ER without specifying subtype (ERα or ERβ). There was no description of positive and negative controls included during the determination of ER-binding sites. Mammary whole mount examinations were conducted on only 4–5 specimens/group. The authors used 1-way ANOVA for statistical analyses; however, because there was only 1 genistein treatment group, 1-way ANOVA would be the equivalent of a *t* test (presumably the genistein and zearalenone data were analyzed separately).

Utility (Adequacy) for CERHR Evaluation Process: The route of administration of treatments rather than oral, and use of only a single dose group thus obviating measurement of dose-response relationships limit the utility of this study. This study has limited utility for the evaluation.

Hilakivi-Clarke et al., 2002 ***(653)***, in a study supported by the American Institute for Cancer Research, American Cancer Society, Komen Breast Cancer Foundation, and DoD, examined the effects of dietary maternal genistein exposure during gestation on development of mammary cancer in adulthood. Sprague Dawley rats were fed a control AIN-93 diet for 7 days upon arrival at the laboratory. A few days prior to mating, the rats (n=17–23 /group) were switched to 1 of 3 AIN-93 diets containing 20% soy isolate, with genistein concentrations of 15, 150, or 300 mg (aglycone equivalent)/kg diet. **[Based on assumed values of rat body weights (0.204 kg) and feed intake (0.02 kg/day)** ***(313)*****, in addition to reported weight gain during pregnancy (~100 g), genistein intake was estimated at 1–1.5, 10–15, and 20–30 mg/kg bw/day.]** Rats fed the medium- and high-dose genistein diets were reported to have serum genistein levels within ranges observed in Asians consuming high-soy diets. Rats were fed their respective diets throughout pregnancy, and after giving birth were fed the control AIN-93 diet. On PND 2 **[day of birth not specified]** female pups from 3 or 4 different litters were fostered to dams from the same dietary group as their mothers. Mammary gland morphology was examined in 3- and 8-week-old female offspring that were not exposed to carcinogens **[number examined not specified]**. At 47 days of age, 23–27 female offspring/group were administered dimethylbenzanthracene by gavage at ~50 mg/kg bw, a dose that induces tumors in ~2/3 animals. In another part of the study, dams (n=36) fed the low-, medium-, or high-dose diets on GD 7–19 were killed on GD 19 and serum 17β-estradiol was determined. Serum 17β-estradiol levels were also measured in offspring (n=5–7/group) at 3 and 8 weeks of age. 17β-Estradiol data were not used for the 8-week-old rats in proestrus because 17β-estradiol levels peak at that stage. Serum 17β-estradiol levels were measured using a double antibody kit. Statistical analyses were conducted using 1- or 2-way ANOVA, Fisher's Least Significant Difference test, chi-squared test, Kaplan and Meier test, or Wilcoxon test.

Genistein had no detected effect on weight gain in dams, length of pregnancy, litter size, or postnatal pup weight gain. Percent successful pregnancy appeared lower in rats fed the high-genistein (55%) than the low- or medium-genistein diets (70–71%), but the effect was not statistically significant. A dose-related increase in serum 17β-estradiol levels was observed in the dams fed genistein, but the results did not attain statistical significance. In offspring of dams fed genistein-containing diets during pregnancy, serum 17β-estradiol levels were not shown to be significantly affected at 3 weeks of age but were significantly reduced at 8 weeks of age in the high-genistein diet group. Morphologic changes in mammary glands of 8-week-old but not 3-week-old offspring of the high genistein diet group included decreased numbers of lobules **[scores of ~3.75, 3.75, and 2.5 in the low-, medium-,**

and high-dose diet groups] and a dose-related increase in terminal end buds **[~30, 45, and 60 in the low, medium, and high dose diet groups]**. Significant effects following dimethylbenzanthracene treatment included increased tumor incidence in the high genistein diet group at 17 weeks (82 versus 67% in the low- and medium- diet groups) and decreased proportion of animals surviving to 17 weeks of age in the medium and high genistein groups (survival 37, 51, and 59% in low-, medium-, and high-dose groups). **[The data table in the study did not indicate statistical significance for the medium-dose genistein group.]** Genistein had no detected effect on tumor latency or multiplicity. The effects of polyunsaturated fatty acids (n-3 or n-6) were also examined in this study, and it was determined that increased levels of polyunsaturated fatty acids in diet were associated with higher levels of 17β-estradiol during pregnancy, more mammary lobules and fewer terminal end buds in offspring, and protective effects against carcinogenicity in offspring. The study authors concluded that *in utero* exposure to genistein could increase breast cancer risk.

Strengths/Weaknesses: A strength of this study is that dosing occurred through the diet, which is the most relevant route for humans. The authors used AIN-93 diet, which has no phytoestrogen activity (per Harlan Teklad). For the genistein-treated groups, diets contained genistein at 1 of 3 dose levels by addition of soy isolate (20% of the diet; 0.075, 0.75, or 1.5 mg genistein **[aglycone equivalent]/g product]**. Dose levels were relevant, as medium- and high-dose levels were reportedly equivalent to Asians consuming a high-dose diet. Female rat pups were cross-fostered on PND 2 to control for environmental factors such as maternal caregiving. A satellite group was included for the measurement of serum 17β-estradiol levels during pregnancy. For serum 17β-estradiol measurements in the offspring, pups were evaluated at 3 weeks of age (sampling time) to ensure that none had undergone vaginal opening prior to collecting a serum sample. For 8-week-old rats, uterine morphology was used to determine estrous stage, and rats were excluded if they were in proestrus. Increased tumor incidence in the high-dose genistein group at 17 weeks corresponded with the increased numbers of terminal end buds and decreased lobule density seen in this group at 8 weeks. A weakness of the study is that it was not clear which group represented the control, as the diets were modified to contain either high or low levels of n-3 or n-6 polyunsaturated fatty acids and low (15 mg/kg diet), medium (150 mg/kg diet), or high (300 mg/kg diet) levels of genistein. None of the groups were maintained on unsupplemented diet. Since it is not possible to feed animals a diet that has no PUFA, the diet that has a total PUFA content similar to laboratory chow may provide a potentially useful reference for the other three diets. Nonetheless, it was not clear if an untreated (genistein) control group existed among the polyunsaturated fatty acids diets, and there was no indication that the genistein-treated groups were compared back to such a control. The source for genistein was given and purity is >98% (as with other studies by other groups that provide the source and where its purity is publicly available). However, there was no mention as to whether the diets were analyzed for concentration, stability, or homogeneity. Maternal blood levels of genistein were not reported. There were no details as to how dams were assigned to treatment groups, and there was no indication that the authors controlled for litter effects. While they cross-fostered pups into different litters (2–3 pups stayed with the biological mother), this cross-fostering would only control for environmental factors. There was no evidence that the authors considered litter of origin when assigning pups to different endpoints. Pregnancy rates were somewhat low for Sprague Dawley rats (e.g., 70% in the low-genistein group), although the sample size was small. Larger sample sizes would have made the 17β-estradiol data more easily interpretable. The authors only measured total 17β-estradiol; it is not known what proportion of this 17β-estradiol existed in a free state.

Utility (Adequacy) for CERHR Evaluation Process: This study assessed the role of *in utero* genistein exposures on several endpoints potentially relevant to this study. The data suggest that *in utero* genistein exposure could act as a tumor promoter the relevance of this animal model to human health is unclear as chemically induced mammary tumors in rats do not recapitulate the pathogenesis or pathophysiology of breast cancer in women. Therefore, this study has limited utility for the evaluation process.

Lamartiniere et al., 1995a, 1995b; Cotroneo et al., 2002; Brown and Lamartiniere, 1995, Brown et al., 1998; Murrill et al., 1996 [323; 650; 654-657], funded in part by NIH, published a series of studies on the role of genistein in mammary carcinogenesis in rats. Sprague Dawley rats were fed standard chow during pregnancy and AIN-76-A, a phytoestrogen-free diet, starting at parturition. **[In 1 study, rats were purchased as weanlings and placed on the AIN-76-A diet *(656)*.]** At birth, litters were culled to 11 pups (4–6 females). During neonatal (PND 2, 4, and 6), prepubertal (PND 16, 18, 20), or pubertal (PND 23, 25, 27, 29) stages of development, female offspring were sc injected with genistein. Doses were equivalent to 500 mg/kg bw in neonates and prepubertal rats and 50 mg/kg bw in pubertal rats. DMSO was the vehicle used for genistein delivery and treatment of controls, with the exception of 1 study that used a sesame oil vehicle *(656)*. Five or more rats per group were evaluated for most endpoints, and at least 19 animals per group were examined for tumor development. Statistical analyses were performed with the Wilcoxon rank sum test, Fisher's exact test, Cochran-Armitage test, Student *t* test, ANOVA, and Tukey test. **[Variances are not always identified in the Results sections of these papers, but the use of SEM appears to be common in this laboratory and is assumed for the data presented below.]**

In rats treated prepubertally with genistein 500 mg/kg bw on PND 16, 18, and 20, total genistein concentrations were measured in serum by HPLC and reported as 4.2 ± 0.6 μM **[1134 ± 162 μg/L]** at 21 days of age and 102 ± 30 nM **[28 ± 8 μg/L]** at 50 days of age *(323)*. Brown *et al.*, 1998 *(657)* noted that the genistein level in the 21-day-old rats was similar to genistein plasma concentrations in infants fed soy formula, 684 μg/L (2.5 μM) *(84)*.

Endocrine parameters were reported for rats treated with 500 mg/kg bw genistein sc during neonatal *(654)* and prepubertal *(323)* stages. A very limited examination of endocrine parameters was included in the study with pubertal treatment with 50 mg/kg bw genistein sc *(656)*. Genistein accelerated female sexual development, as noted by vaginal opening occurring on PND 28 versus 34 in rats treated neonatally and on PND 27 versus 37 in rats treated prepubertally with genistein versus vehicle. Mammary size was transiently increased following treatment with genistein in the neonatal and prepubertal periods. Evaluation of pubertally treated rats at a single time period also revealed increased mammary size. Uterine-ovarian weight was reduced in 21–230-day-old rats treated neonatally, but uterine wet weight was transiently increased in rats treated prepubertally. No significant effects on body weight were observed at any age. Time spent in estrus increased following neonatal genistein exposure (42.9% of the cycle in treated compared to 23.4% of the cycle in control) and prepubertal genistein exposure (36% in treated compared to 23% in control) **[statistical significance not discussed]**. An examination of ovaries fixed in 10% neutral buffered formalin revealed twice as many atretic antral follicles and less than 1/10 the number of corpora lutea **[data not shown]** in 50-day old rats treated as neonates. No significant effects of genistein treatment on the number of oocytes/follicle, atretic follicles, or corpora lutea in ovaries from 50-day-old rats treated during the prepubertal period were detected. RIA measurement of circulating progesterone and 17β-estradiol levels found progesterone

to be significantly reduced **[by 81%]** following neonatal treatment; both hormones were found to be slightly, but not significantly, lower in rats treated during the prepubertal period.

Tumorigenicity following gavage administration of dimethylbenzanthracene at 50 days of age was assessed in rats treated with 500 mg/kg bw genistein during the neonatal *(654; 655)* and prepubertal *(323)* periods. Rats treated with genistein during either developmental period developed only half as many dimethylbenzanthracene-induced tumors as control rats. In neonatally treated rats, genistein significantly increased latency for appearance of palpable tumors (124 ± 33 days compared to 87 ± 37 days in controls) and reduced the incidence of mammary tumors (100 compared to 88% in controls) at 190 days post treatment. No significant difference in time to tumor development in rats treated with genistein during the prepubertal period was detected. Adenocarcinomas represented ≥93% of tumors in all groups of rats.

Effects of genistein on mammary gland development were studied in rats exposed to genistein during each of the developmental periods using whole mounts fixed in 10% neutral buffered formalin; procedures, results, and references are presented in **Table 124**. Consistent effects of genistein treatment included increased numbers of terminal end bud cells in 21-day-old rats and decreased numbers of terminal end bud cells in 50-day-old rats. Numbers of lobule cells were increased in 50-day-old rats treated with genistein during the prepubertal stage and in 90-day-old rats treated with genistein as neonates.

Effects of genistein on proliferation were studied in mammary glands that were fixed in formalin and sectioned *(323; 654; 656)*. As noted in **Table 125** and **Table 126**, evaluations were conducted using PCNA staining (positive cells were described as "cycling") or bromodeoxyuridine incorporation (positive cells were described as "S-phase). **Table 125** summarizes percentages and **Table 126** summarizes the number of cells described as cycling or in S-phase per mammary structure multiplied by the number of structures per gland. Lamartiniere *et al.*, 1995 *(654)* concluded that there were increased numbers of cycling terminal end bud and duct cells in 21-day-old rats treated with genistein as neonates. **[Consistent results were not obtained for 21-day-old rats; results varied according to the evaluation method used (i.e., percentage versus number per structure).]** Increases in cell proliferation from terminal structures were not observed in 22-day-old rats treated during prepubescence but were seen in 30-day-old rats treated during puberty. The authors concluded that genistein exposure in immature animals reduced the number of cycling and S-phase cells during adulthood (50 days of age). **[In some cases no significant effects were seen compared to controls.]**

One study focused on the role of the EGF-signaling pathway in animals treated during the prepubertal period (PND 16, 18, and 20) with 500 mg/kg bw genistein sc *(657)*. Expression of transforming growth factor (TGF)-α, EGF, and EGF receptor in mammary glands of 21- and 50-day-old rats were examined using RT-PCR, Western blots, and immunohistochemical techniques. The study authors noted that in terminal ductal structures of 21-day-old rats, TGF-α and EGF receptor protein, but not mRNA expression, increased. **[Based on immunohistochemistry data, the effect was statistically significant only for EGF receptor.]** In mammary terminal structures from 50-day-old rats, mRNA expression was down-regulated for TGF-α during proestrus and estrus and EGF during proestrus. The study authors stated that in 50-day-old rats, immunostaining intensity was decreased for EGF receptor in terminal end buds and increased for EGF in terminal end buds and terminal ducts. **[Results for EGF were not statistically significant.]**

Table 124. Effect of Genistein Treatment in Rats on Development of Mammary Structures

Treatment, PND	Dose, mg/kg bw	Evaluation PND	Terminal End Buds	Terminal Ducts	Lobules				Reference
					Type I	Type II	Type III	Total	
2, 4, 6	500	21	↑	↑	↔	↔			(654)
		50	↓	↔	↔	↔			
2, 4, 6	500	50	↓	↔	↔	↔	↔		(655)
		90	↔	↔	↔	↔	↑		
16, 18, 20	500	21	↔	↔	↔				(650)
16, 18, 20	500	21	↑	↔					(657)
		50	↓	↓				↑	
16, 18, 20	500	22	↔	↓	↔	↔			(323)[a]
		33	↔	↔	↔	↔			
		50	↓	↔	↔	↑			
23, 25, 27, 29	50	30	↔	↔	↑				(656)

[a] Values for this study were presented as percentage instead of numbers.
↑, ↓, ↔ Significantly increased, decreased, or no change compared to control.

Table 125. Percentages of Mammary Cells Cycling or in S-phase Following Genistein Treatment of Rats

Treatment, PND	Dose, mg/kg bw	Evaluation PND	Terminal End Buds		Terminal Ducts		Type I Lobules		Type Ii Lobules	
			Cycling	S-Phase	Cycling	S-Phase	Cycling	S-Phase	Cycling	S-Phase
2, 4, 6[a]	500	21	↓	↓	↔	↔	↓	↔		
		50	↔	↓	↔	↓	↔	↔	↔	↓
23, 25, 27, 29[b]	50	30	↑	↑	↑	↑	↑	↑		

[a]Lamartiniere et al., 1995 *(654)*; [b]Brown and Lamartiniere, 1995 *(656)*.
Cycling=PCNA positive; S-phase=BrdU positive.
↑,↓,↔ Significantly increased, decreased, or no change compared to control.

Table 126. Numbers of Mammary Cells Cycling or in S-phase Following Genistein Treatment of Rats

Treatment, PND	Dose, mg/kg bw	Evaluation PND	Terminal End Buds		Terminal Ducts		Type I Lobules		Type Ii Lobules	
			Cycling	S-Phase	Cycling	S-Phase	Cycling	S-Phase	Cycling	S-Phase
2, 4, 6[a]	500	21	↑	↔	↑	↑	↓	↔		
		50	↓	↓	↔	↓	↔	↓	↔	↓
16, 18, 20[b]	500	22		↔		↓				
		33		↔		↔		↔		
		50		↓		↔		↔		↑
23, 25, 27, 29[c]	50	30	↑	↑	↔	↔	↑	↑		

[a]Lamartiniere et al., 1995*(654)*; [b]Murrill et al., 1996 *(323)*; [c]Brown and Lamartiniere, 1995 *(656)*.
Cycling=PCNA positive; S-phase=BrdU positive.
↑,↓,↔ Significantly increased, decreased, or no change compared to control.

APPENDIX II
Developmental Toxicity of Soy Formula

A subsequent study further examined mechanisms of prepubertal genistein exposure on mammary glands of 21-day-old rats *(650)*. Consistent with the earlier study by Brown *et al.*, 1998 *(657)*, prepubertal genistein treatment increased EGF receptor protein expression in mammary glands. Although phosphorylated-EGF receptor expression was increased, normalization to total EGF receptor resulted in no detected difference, indicating no net effect on phosphorylation. Expression and phosphorylation of downstream EGF receptor targets were not affected **[data not shown]**. Genistein treatment also increased progesterone receptor expression and decreased staining intensity of ERα in mammary glands. Effects in genistein-treated rats were similar to those in estradiol benzoate-treated rats. Treatment with the anti-estrogen ICI 182,780 inhibited genistein and estradiol benzoate effects on mammary development and inhibited expression of EGF and progesterone receptors; the ICI 182,780 effects led authors to suggest blocking of ER function. Similar effects on progesterone expression **[data not shown]** and EGF receptor expression in intact and ovariectomized rats suggested no indirect action of genistein via increased circulating 17β-estradiol. The study authors concluded that genistein acts via an ER-based mechanism to stimulate mammary gland proliferation and differentiation, which may protect against mammary cancer.

In conclusion, the studies from the laboratory of Lamartiniere were interpreted by the authors as suggesting that acute sc exposure of immature animals to 500 mg/kg bw genistein resulted in increased differentiation of immature terminal end buds, leading to a greater number of lobules, thought to be more resistant to carcinogens, during adulthood. It appeared that the effects were mediated through ERs, which regulate progesterone receptor and EGF receptor. Up-regulation of EGF receptor in immature rats did not occur through tyrosine phosphorylation. EGF receptor was down-regulated in adult rats, and the authors hypothesized that a less active EGF-signaling pathway in adulthood suppressed mammary cancer development *(658)*.

Strengths/Weaknesses: The studies address an important and relevant subject. The timings of exposure studied are relevant and clear. Attempts to understand mechanism are a strength. Statistical analyses are appropriate and studies appear powered for most endpoints. Serum measures of genistein were obtained in some studies. For the DMBA studies, PND 50 is within the most sensitive window most widely used with DMBA (PND 21 could be used with NMU as the carcinogen). The use of a lab chow during pregnancy will have exposed dams and fetuses to highly variable genistein exposures during a developmentally responsive period. The prenatal diets also do not control for sources or levels of other micro/macronutrients that could affect the mammary glands. Starting the isoflavone free diets at parturition will likely not account for highly variable phytochnical exposures during the developmentally sensitive period *in utero*. The route of administration is not relevant to human exposures. Dose(s) is not relevant to human exposures, probably even for supplement usage. Regimen used in several studies is not relevant to how humans are exposed. Dose solutions were not consistently analyzed for stability, concentration, or homogeneity; authors also do not consistently control for litter effects.

DMBA dose is high and will not detect increased tumorigenesis; thus, data and overall interpretations may be biased towards detecting only protective (or apparently no-effect) outcomes. The mechanistic studies are potentially compromised by the high dose regimen used. Several of the targets identified/studied have been studied in detail in multiple models; some are no longer thought to be relevant mechanisms for dietary or supplement exposures in humans (e.g., the EGFR tyrosine kinase). For EGFR, the data are inconsistent and downstream markers of activity (or net EGFR phosphorylation) are unchanged, suggesting that it is not a mechanistically important target even at the high exposures

used. For the more relevant targets, these studies seem to add little to what was already known or proposed (with respect to ER as a mechanism). The weaknesses in these studies outweigh the strengths.

Utility (Adequacy) for the CERHR Evaluative Process: These studies are of no utility to the evaluation process. If it is subsequently determined that the serum levels are relevant to humans (despite the regimen), some of the individual studies would have limited utility if they also used an appropriate diet (which seems likely in some of the studies included in this group).

Lamartiniere et al., 2002 ***(659)*** supported by the National Institute of Health, examined the potential of genistein in the diet to protect against breast and prostate cancers. The paper presents the results of five previously published studies: three studies with female rats, one study with male rats and one study with a male transgenic mouse model. **[Detailed methods and results are not reported.]** For the male studies, no new data is presented and no new conclusions are drawn. For the females, no new data is presented, but the tumor multiplicity data from the three studies are combined and a new conclusion is drawn. In all three female studies, the rats were exposed to diets containing genistein or a control diet (AIN-76A); but each study had a different exposure regimen. On postpartum day 50, the females were treated with 80 mg/kg dimethylbenz[a]anthracene (DMBA) to induce mammary tumors, and killed on postpartum 180. **[The day of sacrifice was only stated for one study, assumed to be the same for all three.]** The combined data is presented in **Table 127**:

Table 127. Dietary Genistein – Timing of Exposure and Mammary Cancer Chemoprevention (Lamartiniere et al., 2002)

Exposure, 250 mg genistein/kg diet	*Relative Mammary Tumor Multiplicity*
No genistein	8.9
Prenatal genistein (throughout gestation)	8.8
Adult genistein (after 100 days of age)	8.2
Prepubertal genistein (postpartum day 1 – 21)	4.3
Prepubertal and adult genistein	2.8

From Lamartiniere et al., 2002 *(659)*.

Authors' conclusion: Mammary cancer chemoprevention was demonstrated after prepubertal and combined prepubertal and adult genistein treatments, but not after prenatal or adult-only treatments, demonstrating that the timing to exposure to genistein is important for mammary cancer chemoprevention.

Strengths/Weaknesses: This study summarizes the results of several well designed studies conducted by this laboratory group in which rats were treated with genistein or a control diet. This is a review of prior work from this laboratory. It is not clear that the data have been combined in a statistically appropriate manner, e.g., as would be required for a statistical meta analysis. Any new interpretations of data combined across studies without use of appropriate statistical methodology may be unreliable. The main strength of this report is the new comparison of tumor multiplicity in female rats from several similar studies but with different periods of genistein treatment. A weakness of this is approach is that the exposures were not done in a single study which would obviate the potential for changes or variations in study diets and methods within the lab. The lack of methodological details in several

studies makes interpretation of the data problematic. The route of administration of genistein is not relevant to humans. Finally, the animal model used although valuable for identification of potentially important mechanisms of mammary tumorigenesis is of limited value to human breast carcinogenesis.

Utility (Adequacy) for the CERHR Evaluative Process: This study has no utility for in the evaluation.

Pei et al., 2003 ***(660)***, in a study supported in part by the Ministry of Health, Labor, and Welfare of Japan, examined the effects of prenatal and prepubertal genistein exposure via sc injection on chemically induced carcinogenesis in the rat. Pregnant and lactating Sprague Dawley rats were fed NIH-O7, a phytoestrogen-free diet. During pregnancy, 5–6 rats/group were sc injected with genistein (>99% purity) at 0 (DMSO vehicle), 1.5, or 30 mg/kg bw/day on GD 15–19 (day of vaginal plug not defined). A total of 26–30 female offspring were produced in each group. Thirty female rats/group from additional untreated dams **[number of dams from which female offspring were obtained was not specified]** were sc injected with genistein 1.5 or 30 mg/kg bw/day from 15 to 19 days of age. The low dose was reported to be equivalent to genistein intake in Asian populations (0.4–1.5 mg/kg bw/day). Vaginal opening was monitored daily, and body weights were measured weekly. Six randomly selected rats from each dose group were killed at 28 days of age to examine mammary gland histopathology, and numbers of ERα-, progesterone receptor-, p63- (involved in cell renewal), and PCNA-positive cells by immunohistochemistry methods. The remaining 28-day-old rats from each group (~20–24/group) were ip injected with 50 mg/kg bw N-methyl-N-nitrosourea dissolved in 0.5% acetic acid. Rats were palpated weekly for mammary tumors. Estrous cyclicity was monitored from 10 to 14 weeks of age. Rats were killed at 26 weeks of age for evaluation of mammary tumors, ERα- and progesterone receptor-containing cells, and cell proliferation. Mammary carcinomas with more than 80% ERα- or progesterone-positive cells were classified as hormone-dependent. Tumor incidence data were analyzed by Mantel-Cox Log rank test. Estrous cycle and hormone-dependency data were assessed by chi-squared test. All other data were analyzed by ANOVA, Kruskal-Wallis test, Fisher's Least Significant Difference test, or Bonferroni-Dunn test.

Results are summarized in **Table 128**. Prenatal genistein treatment resulted in lower body weights, while prepubertal genistein treatment resulted in higher body weights compared to controls on PND 28. Relative (to body weight) uterine-ovarian weights were lower in both dose groups treated prenatally and in the low-dose group treated in the prepubertal period. There were no detected histopathological changes in ovaries or uteri at 28 days of age. Vaginal opening was accelerated in rats treated with genistein 30 mg/kg bw/day during the prepubertal period **[mean day of vaginal opening was not reported by study authors]**. All untreated rats had normal 4–5-day estrous cycles, but percentages of rats with either 3-day or 6-day estrous cycles were increased in all treated groups. The estrous phase of the cycle was prolonged and the diestrous phase was shortened in rats treated with genistein during prepuberty. At 28 days of age, mammary gland development was comparable in all treatment groups, with similar numbers of terminal end buds at the periphery of the mammary tree. Genistein-treated rats had decreased numbers of ERα-, progesterone-, PCNA-, and p63-positive mammary terminal end bud cells. Genistein treatment decreased the number of rats with carcinomas ≥1 cm, but statistical significance was attained only in the group given 1.5 mg/kg bw/day during prepuberty. Genistein had no detected effect on tumor multiplicity, latency, or numbers of hormone-dependent carcinomas. The majority of tumors (91–100%) in the control and all dose groups were hormone-dependent. The study

Table 128. Effects in Rats Treated with Genistein During Prenatal or Prepubertal Development (Pei et al., 2003)

Parameter	*Genistein Doses, mg/kg bw/day*			
	During Gestation		*In Prepuberty*	
	1.5	*30*	*1.5*	*30*
Body weight at PND 28	↓13%	↓10%	↑28%	↑25%
Relative (to body weight) uterine-ovarian weight at PND 28	↓23%	↓32%	↓20%	↔
Percentage of rats with 3-day estrous cycles [a, b]	~10%	~5%	~8%	↔
Percentage of rats with 6-day estrous cycles [a, b]	↔	~10%	~10%	~17%
Percentage of time in estrus	↔	↔	↑20%	↑34%
Percentage of time in diestrus	↔	↔	↓19%	↓22%
Percentage of terminal end bud cells at PND 28 postive for:				
ERα	↓13%	↓28%	↓30%	↓25%
Progesterone	↓12%	↓29%	↓27%	↓27%
p63	↓17%	↓15%	↓12%	↓17%
PCNA	↔	↓6.3%	↓11%	↓14%
Percentage of rats with carcinomas ≥ 1 cm	↔	↔	↓40%	↔

↑, ↓, ↔ Significant increase, decrease, or no change.
[a] Values estimated from a graph by CERHR.
[b] All control rats had normal (4–5 day cycles); statistical significance of the effect was not determined.
From Pei et al., 2003 *(660)*.

authors suggested that prepubertal exposure to genistein protects rats against N-methyl-N-nitrosourea-induced mammary carcinomas by reducing levels of ERα-, progesterone receptor-, p63-, and PCNA-positive cells.

Strengths/Weaknesses: Strengths include the use of a diet free of phytoestrogens, purity of genistein >99%, use of multiple dose levels, and selection of physiological (1.5 mg/kg bw/day) and pharmacological (30 mg/kg bw/day) dose levels, although a relevant route of exposure was not used. The NIH-07 diet usually contains soybean meal as a protein source; not clear how this was reformulated to be phytoestrogen-free. The use of two doses of genistein is a strength; source and purity of genistein are provided. Weaknesses include the lack of analysis of dose solutions for concentration verification, stability, or homogeneity, the lack of detail on how dams and pups were assigned to treatment groups, and the lack of indication that the authors controlled for litter effect either in their sampling methodology or statistical analyses. NMU regimen will not easily detect increased tumorigenesis; thus, data and overall interpretations may be biased towards detecting only protective (or apparently no-effect) outcomes. The definition of hormone-dependence based on 80% ER+ cells is weak.For the prepubertal treatment, 60 female offspring of mothers not exposed to genistein received sc injections, indicating that the prepubertal genistein treated animals came from different litters than those used in the prenatal experiments. There was no information on the number of litters from which these pups originated or that any litters were culled to standardize growth rates. Furthermore, it does not appear as if there was a concurrent prepubertal vehicle control group. It was difficult to determine how genistein treatment affected body weights because the authors did not report any body weights prior to 3 weeks of age. This lack of reporting was particularly

problematic for the prepubertal animals, which may have weighed more than the control animals prior to genistein treatment. Genistein-treated rats reportedly had lower relative uterine-ovarian weights; however, the reason for this decrease was not given and is difficult to discern given that body weights were different across the treatment groups. Vaginal opening was accelerated in the prepubertal 30 mg/kg bw/day genistein-treated animals, but growth rate was not considered in this determination. Furthermore, historical control data were not presented for either age at vaginal opening or estrous cyclicity. The authors stated, "The number of tumors per rat (tumor multiplicity) was low in groups 2, 4, and 5…" when tumor multiplicity (study Table IV) in group 2 did not differ from the control group (mean=1.6 in both groups). The impact of body weight differences on the various endpoints was not known.

Utility (Adequacy) for CERHR Evaluation Process: Although the authors used doses of genistein that produced circulating levels representative of humans consuming a phytoestrogen rich diet the route of administration is unsuitable for direct translation of study results to humans. However, the study results provide insight into potentially important mechanisms of genistein action on mammary gland development. However, the lack of prepubertal vehicle control and suitable positive and negative controls limit the utility of this study. Therefore, it is concluded that this study has no utility for the evaluation process.

Su et al., 2007 ***(661)***, funded by the United States Department of Agriculture, investigated the influence of gestational exposure to genistein on potential protection against tumorigenesis initiated by the direct-acting carcinogen *N*-methyl-*N*-nitrosourea (NMU), and examined potential mechanisms underlying these effects. Time-mated Sprague Dawley rats were received from CRL: Wilmington, MA. On gestation day 4 they were randomly assigned to semi-purified isocaloric diets (AIN-93G) with corn oil substituting for soybean oil and containing the following: i) casein (CAS), ii) CAS to which genistein (GEN) was added in the aglycone form (250 mg/kg feed), or iii) soy protein isolate (SPI). The latter contained 394 $\pm$ 16 mg total isoflavones/kg diet (216 mg/kg GEN and 160 mg/kg daidzein). **[number of dams per group not stated, bedding not described]** At delivery (PND 0), each dam was assigned 10 pups. The dams that had been fed CAS throughout pregnancy continued on the CAS diet (CAS group - negative control); the dams that had been fed SPI were divided into two diet groups: one group continued on SPI (SPI group - positive control), the other group was switched to the CAS diet (SPI-CAS group); all dams that had been fed the GEN diet were switched to the CAS diet (GEN-CAS group). After weaning on PND 21, pups remained on the diet of their dams during lactation. At PND 50, F_1 females (n=10 each for CAS, SPI, and SPI-CAS; n=5 for GEN-CAS) were killed and inguinal mammary gland pair #4 was saved. The left gland was fixed for paraffin embedding and the right gland was homogenized in TriZol for RNA extraction. On PND 51, the remaining F_1 females were administered NMU at a dose of 50 mg/kg body weight by intra venous injection. Rats were weighed weekly and, beginning 2 weeks after NMU injection, were palpated weekly for mammary tumors. Rats from all diet groups were killed when 80% of CAS-fed rats showed palpable tumors (PND 149, 99 days after NMU injection). Tumors were weighed and preserved in NBF. Sections of the largest tumor from each tumor-bearing rat were stained with H&E and examined histologically and immunohistochemistry was performed on sections of mammary gland. Data, presented as least square means were subjected to analysis by one-way analysis of variance and further evaluated by Tukey's test. Differences between percentage values for tumor parameters were analyzed by Fisher's exact test. $P \leq 0.05$ was considered statistically significant.

Table 129. Summary of the Effect of Diet on Tumorigenesis (Su et al., 2007)

Effect	*Diet*			
	CAS (n=47)	*SPI (n=44)*	*SPI-CAS (n=47)*	*v*
Post-NMU day that 50 % of rats had tumors	63	78	77	71
Tumor incidence (% of rats with a tumor)	81%	61%	85%	82%
Rats with more than 7 tumors (% of rats)	45%	20%	2%	20%
Invasive ductal carcinomas (% tumor-bearing rats)	53%	67%	30%*	50%

*Statistically significant.
From Su et al., 2007 *(661)*.

Table 129 presents a summary of the effect of diet on tumorigenesis. The appearance of palpable tumors occurred earliest with the CAS group. At the conclusion of the study (PND 149), the SPI group had ~20% less tumors than the other diet groups. There were lower tumor numbers per tumor bearing rat in the SPI-CAS group relative to the other diet groups. The incidence of benign and precancerous lesions did not differ between diet groups; the diet groups also showed a comparable incidence (percentage) of tumors designated as cancerous lesions. The SPI group showed the highest percentage of invasive ductal carcinomas (statistically significant), whereas the SPI-CAS group had the lowest percentage.

Mammary terminal end buds (TEBs) of PND 50 rats in the SPI group had a significantly higher number of cells showing $Tp53^{Ser46}$ immunoreactivity relative to those of the other diet groups. Ductal epithelium structures showed minimal immunostaining for $Tp53^{Ser46}$ (not statistically significant). The transcript levels of the pro-apoptotic genes Bax, p21 and PTEN did not differ between groups. Expression of PTEN in TEB was significantly higher for the SPI group compared to the SPI-CAS and the GEN-CAS groups; a similar trend was seen in the ductal epithelium (DE). E-cadherin localized at sites of cell-cell contacts in mammary TEB and DE of rats of the four diet groups showed greater membrane E-cadherin immunoreactivity in SPI-CAS and GEN-CAS groups relative to SPI and CAS groups respectively.

Authors' conclusion: Limited exposure during gestation to soy protein isolate (SPI) elicited significant mammary tumor protective effects that may have increased advantage over that of lifetime exposure. Exposure to supplemental GEN at a similar developmental window does not result in mammary tumor protection comparable with that of SPI. Signaling pathways were identified that involve the tumor suppressors PTEN and E-cadherin as potential mechanisms underlying the tumor protective effects of dietary soy proteins at two developmental exposure contexts.

Strengths/Weaknesses: Strengths of this study include use of dietary exposure to soy protein isolates, use of AIN-based semi-purified (isoflavone-free) basal diet, and the gestational timing of exposure is relevant. Source of genistein (Sigma) is provided but not the purity; however, it is reasonable to assume this is ≥98% pure (per Sigma). Appropriate pathology performed on tumors. Animals were randomly assigned to each group; may have created "unequal" groups (e.g., body weight distribution). The casein-genistein group data difficult to evaluate without a genistein-genistein control. It was not clear that litter was the unit for analysis. Sacrifice on PND 50 or treatment with NMU to induce

tumors and sacrifice on PND 149 allowed for assessment of signaling pathways potentially important in tumorigenesis. However, the timing (PND 51) and dose of NMU appear optimal for maximum tumorigenicity, and so will not easily detect increased tumorigenesis (tumor incidence ≥80% in control diet); thus, data and overall interpretations may be biased towards detecting only protective (or apparently no-effect) outcomes. In addition, although NMU is a well established model of mammary gland tumorigenesis the relevance of this model for human breast cancer development is unclear. Assessment of molecular markers thought to be important in breast cancer carcinogenesis is a strength of the paper but the authors did not include endpoints that would also have been useful to assess effects of the diets on development, only the expression of selected genes and apoptosis were measured in the non-carcinogen exposed mammary glands. In addition, the authors did not look at latency using standard survival analysis methods.

Utility (Adequacy) for CERHR Evaluation Process: This study has no utility in the evaluation.

Wang et al., 2006 ***(662)*****,** supported by the American Chemistry Council, evaluated mammary responses to pre- and postnatal dietary genistein and methoxychlor in adult female and male rats and investigated relevant mechanisms. Pregnant female Sprague-Dawley rats (CRL, Raleigh, NC) were randomly assigned to treatment groups on gestation day 0: genistein 300 ppm, genistein 800 ppm, methoxychlor 800 ppm, genistein 300+methoxychlor, genistein 800+methoxychlor, **[the level of methoxychlor in these combination groups was not clearly stated, but based on the discussion text, it appears to be 800 ppm]** or control diet **[control diet was not described, presumed to be the base diet] [purity was not stated for any substance]**. The prescribed amounts of genistein and methoxychlor were blended with a base diet (custom soy- and alfalfa-free diet); genistein concentrations were relevant to animal and human exposures under various dietary scenarios, the methoxychlor dose was chosen to elicit significant toxicological responses and is not expected to represent environmental exposures. The dams were fed the prepared diets from GD 0 through gestation and lactation (n=2 dams per group); alpha dry cellulose bedding was provided. After weaning on PND 22, the offspring remained on the same diet as their dams. On PND 90, all rats were killed and trunk blood and inguinal mammary glands were collected. One mammary gland from one male and one female per litter was prepared for whole mount preparations (n=4 glands per group), the other inguinal mammary gland from these animals and the inguinal mammary gland from three males in each group were prepared for histological evaluation and immunohistochemistry. Concentrations of serum hormones were determined and analyzed by two-way analysis of variance with genistein and methoxychlor each as a treatment factor. Immunoblotting of the mammary tissue was conducted. Microarray procedures were used to determine gene expression in the mammary gland tissue samples **[no statistical analysis described for these data]**.

<u>*Males*</u>: Compared to controls, male rats in all treated groups displayed greater total glandular areas. Males receiving genistein at both 300 and 800 ppm had moderately greater glandular size and glandular tissue density. Markedly different changes were seen for males in the genistein 800 ppm+methoxychlor group. In tissue sections of control rats, sexual dimorphism of the mammary morphology was evident. In methoxychlor-treated male rats, the mammary tubuloalveolar acini were greater in number and larger in volume, the ducts were elongated and the ductal walls were lined with more layers of epithelial cells, and the alveoli were larger and greater in number, with lumens visible. The genistein treatment had limited effects on ductal elongation and epithelial hyperplasia. More prominent alveolar proliferation was observed in the animals treated with genistein at 800 ppm

than at 300 ppm. Immunohistochemical staining for PCNA revealed a greater number of proliferating cells in the group receiving genistein 800 ppm+methoxychlor compared to controls.

Females: Mild ductal and alveolar proliferation was observed in females treated with methoxychlor; when exposed to both compounds, the glandular tissue also contained varying degrees of fibrosis. There was no statistically significant difference in estradiol, testosterone, GH, FSH, LH or prolactin concentrations following either genistein or methoxychlor treatment. Animals in the genistein 800 ppm group had significantly lower serum IGF-1 concentrations.

Authors' conclusion: The results indicated that treatment effects are likely due to interactions between steroid hormone receptor-mediated signals and growth factor-driven cellular pathways. The distinctive responses associated with the genistein+methoxychlor combination were likely linked to enhanced actions of insulin-like growth factor 1 and related downstream pathways.

Strengths/Weaknesses: This appears to be a subset analysis of the You, *et al.*, 2002 studies with several of the same strengths and weaknesses. A major strength of this paper is the use of specifically formulated diet for the delivery of genistein and methoxychlor. Exposure to the test diets throughout gestation, lactation, and post weaning is a strength of the present study although lifetime exposure is not necessarily relevant to the assessment of soy formula. The concentrations used provide circulating levels that are representative of human exposure to phytoestrogen rich diets. The rational for terminating the animals at 90 days of age is unclear. Measurement of circulating gonadal steroids and pituitary hormones may not be sensitive enough to pick up changes in signaling pathways that are important in mammary gland development. Absence of any data on reproductive function is a weakness of the study. Formulation of the base diet may not adequately control for the sources and concentrations of micronutrients that could interact with the genistein and/or methoxychlor treatments (it does appear to be a soy/isoflavone free diet). Purity of the dietary components is not provided. The lack of culling could lead to litters of different sizes and different exposures/pup. From the You *et al.*, 2002 studies, at PND 21, female body weights in the 800 genistein diet are significantly lower than in controls, potentially indicative of toxicity; lack of 800 genistein effect on uterus weight is inconsistent with their previous study, Casanova *et al.*, 1999 *(634)*. Microarray studies were reported. The samples were not pooled, which is a strength, but the data analysis methods are weak and the number of samples studied is too small for the data to be meaningful.

Utility (Adequacy) for CERHR Evaluation Process: This study has limited utility for the evaluation.

Yang et al., 2000 ***(663)***, supported by the Japanese Private School Promotion Foundation, evaluated the effects of prenatal exposure of Sprague Dawley rats to genistein via sc injectin on subsequent susceptibility to methylnitrosourea-induced mammary cancer. Genistein (>99% purity) in DMSO was given sc at 5 or 25 mg/kg bw/day on GD 16–20 (plug day not specified). An untreated control was used. Female offspring of the untreated control dams were injected sc with genistein in DMSO at 0 or 12.5 mg/kg bw/dose on PND 15 and 18 (birth=PND 0). **[No information was provided on culling, weaning, or litter allocation of postnatally treated animals.]** On PND 35, 4–9 females per dose group were killed, and thoracic mammary glands were fixed in formalin and prepared for whole-mount evaluation after staining with hematoxylin. The remaining females were treated with methylnitrosourea 50 mg/kg bw ip. Vaginal cytology was used to monitor the estrous cycle from 12

to 16 weeks of age. Animals were examined weekly for palpable breast tumors and were killed when the largest tumor reached 1 cm diameter or at 35 weeks of age. Mammary tumors and abdominal mammary glands were fixed in neutral buffered formalin, embedded in paraffin, sectioned at 4 μm, and stained with hematoxylin and eosin for light microscopy. Immunohistochemistry was used to evaluate tumors for proliferation (using antibody to PCNA) and estrogen and progesterone receptor using counts of antigen-positive cells among at least 1000 cells from 5 different tumor sections. Tumors containing more than 80% ER- or progesterone receptor-positive cells were considered hormone-dependent. Data were analyzed using chi squared and the Mann-Whitney *U* test. **[There is no indication that litter of origin was considered in the analysis.]**

There were no detected effects of treatment on birth weight, survival, or general health of dams and pups **[data were not presented]**. PND 35 body weight was significantly lower among offspring exposed to genistein either pre- or postnatally (11–18% lower than the untreated control). Relative uterine-ovarian weight was described as decreased in genistein-exposed offspring. **[Marked in the data table as statistically significant for the group in which dams received genistein 5 mg/kg bw/day, but numerically lower in the other groups. The Expert Panel believes the lack of a dose-response relationship in the statistical analysis may be due to the use of 8 offspring in the 5 mg/kg group and 4 offspring in the other genistein-exposed groups.]** Evaluation of estrous cycles in 18–29 females/group showed a statistically significant increase in mean cycle length in animals prenatally exposed to maternal genistein at 5 mg/kg bw/day (0.4-day increase) and postnatally exposed to two 12.5-mg/kg bw doses of genistein (1.4-day increase). There was a significant increase in mean time/cycle spent in estrus in all genistein-exposed animals (0.2–0.8 days).

On PND 35, there were no qualitative differences in the appearance of mammary gland tissue in genistein-exposed or untreated animals. Immunohistochemistry assessment of proliferation, ER, and progestin receptor did not show a treatment effect. There was no detected treatment effect on the number of rats developing mammary tumors >1 cm or on the latency from methylnitrosourea treatment to recognition of a tumor >1 cm. The mean (± SEM) number of mammary tumors (including those identified histologically) per animal was statistically increased in animals from dams treated with genistein 5 mg/kg bw/day (2.9 ± 0.5) compared to untreated animals (1.5 ± 0.2, $P<0.05$). The mean number of tumors per animal in the group exposed prenatally to 25 mg/kg bw/day to the dam (2.6 ± 0.5) was not statistically different from the control rate according to the authors **[$P=0.026$, *t* test performed by CERHR]**. Most tumors >1 cm were hormone-dependent; no significant difference was detected in the proportion of hormone-dependent tumors by treatment group. The authors concluded that short exposure to genistein during the perinatal period in rats increased susceptibility to methylnitrosourea-induced mammary tumors as manifested by an increase in the number of tumors per rat.

Strengths/Weaknesses: A strength of this study is that rats were exposed to multiple dose levels of genistein, which allowed some assessment of dose-response relationships. Large numbers of cells (1000 cells from 5 different areas of each tissue section) were counted during immunohistochemistry experiments to identify ER-, progesterone receptor-, and PCNA-positive cells. Offspring body weights were monitored throughout the experiment. A weakness is that the acclimation period for this study was very short; rats were received on GD 14 and injections began on GD 16. It was not specified whether the rats received genistein-free chow. Dose solutions were not analyzed for concentration, stability, or homogeneity. There was only one dose level used for PND 15 and 18 exposures, which did

not allow an assessment of dose-response relationships. Maternal/fetal blood levels of genistein were not reported. The day on which the dams were sperm-positive was not identified (GD 0 or 1).There were no details as to how dams were assigned to treatment groups, and there was no indication that the authors controlled for litter effects. Lack of culling and/or cross-fostering seems to be a significant issue with this study. There was no evidence that the authors considered litter of origin when assigning pups to different endpoints. Litter data (e.g., number of litters, litter size, pup body weights) were not presented. Estrous cycle evaluations were performed at 12–16 weeks of age after exposure to methylnitrosourea at 35 days of age, which could have contributed to altered cycles. Mammary whole mounts were prepared from 4–9 females/group at 35 days of age; a sample size of 4 is small for such an assessment. The authors did not mention whether negative or positive controls were used during their immunohistochemistry experiments; thus, it is not possible to confirm the specificity of labeling although this was not a key endpoint of the study. There was a significant decrease in relative uterine-ovarian weight in 35-day-old rats exposed to genistein 5 mg/kg bw/day on GD 16–20; however, there was no indication that the authors controlled for estrous cycle stage at the time of sample collection on PND 35. The relative uterine-ovarian weights in the 12.5 and 25 mg/kg bw/day dose groups did not achieve statistical significance, which may have been related to the small sample sizes in these groups (n=4). **I agree – several of the key endpoints appear to be underpowered** The 25 mg/kg bw/day group had a greater decrease in uterine-ovarian weight than the 5 mg/kg bw/day dose group. According to study Table 2, the control value for length of 1 estrous cycle was 42 ± 0.1 days (presumably, this should be 4.2 ± 0.1 days). While statistically significant, it is difficult to discern the biological significance of a 4.2-day estrous cycle in control animals compared to a 4.6-day cycle in animals treated *in utero* with 5 mg/kg bw/day genistein, given that normal estrous cycles are 4–5 days in length and the authors did not control for litter effects. A similar issue applies to length of estrus, which was 1.1 days in control animals compared to 1.3 days in animals treated with genistein 25 mg/kg bw/day (normal duration of estrus is 1–2 days). The "increased time in estrus" did not exhibit a dose-response relationship. With *in utero* genistein exposure, neither the percent increase in mammary carcinoma incidence nor the mean number of mammary carcinomas/rat followed a dose-response relationship.

Utility (Adequacy) for CERHR Evaluation Process: This study has no utility for the evaluation process for several reasons. Of note, injection is not considered a relevant route of exposure, failure to account for litter effects in the analyses, and the limited sample size for some outcomes make interpretation of the data difficult. The relevance of changes in some outcome measures to human health is unclear. For example, the biological significant of a small although significant change in estrous cycle length (mean change of 0.4 days) is unknown.

You et al., 2002 ***(644)***, supported by CIIT, evaluated the developmental effects on the rat mammary gland of dietary genistein during gestation and postnatally, alone and in combination with methoxychlor, a pesticide with the estrogenic metabolite HPTE. **[The animals in this study are a subset of the animals reported in *(633)* (L. You, personal communication, February 2, 2004).]** Time-mated Sprague Dawley rats were obtained on GD 0 (the day sperm were found in the vaginal smear). Animals were randomized by weight to 1 of 6 groups (8 animals/group). A control group was given untreated feed (a soy- and alfalfa-free diet). Treated animals were given the same feed, with the addition of genistein (>98% pure), methoxychlor (~95% pure), or both. The 5 diet combinations were: 800 ppm methoxychlor; 300 ppm genistein; 800 ppm genistein; 300 ppm genistein + 800 ppm methoxychlor; and 800 ppm genistein + 800 ppm methoxychlor. The 300 ppm dose of genistein was

selected to approximate the amount of genistein in the NIH-07 rodent diet. The 800 ppm doses of genistein and methoxychlor were both based on previous studies showing endocrine effects at these exposure levels. **[For information on feed consumption, body weight, and estimated genistein ingestion, see the discussion of You et al., 2002 *(633)* in Section 3.2.1.4.]**

Dams were maintained on their assigned diets during pregnancy and lactation. **[No statement was made about culling. The authors note that pups would likely have ingested treated feed during the last part of the lactation period.]** On PND 22, pups were killed. One pup/sex/litter had inguinal mammary glands removed for evaluation. In 4 animals per treatment group **[probably 4/sex/group based on the study Results section]**, 1 gland was used for whole mount preparation, and the other was used for tissue section. Whole mount mammary glands were evaluated using computerized image analysis for total gland area and the number of terminal end buds and lateral buds. Immunohistochemistry studies were performed on fixed mammary gland sections from male offspring using antibody to insulin-like growth factor (IGF)-1 receptor-β, ERα, progesterone receptor, and PCNA. PCNA-stained slides were used to derive a labeling index, which was the ratio of actively dividing cells to total cells in the section. Trunk blood was collected for measurement of IGF-1 and prolactin by RIA. **[The results section indicates 3 or 4 animals per group.]** Statistical analysis was by 3-way ANOVA (sex, methoxychlor, and genistein) for whole mount data and 2-way ANOVA (methoxychlor and genistein) for serum hormone measurements and immunohistochemistry (which were only performed on males). Post-hoc *t* testing was used when ANOVA suggested an effect of genistein.

Offspring in the control group had inguinal mammary glands described as rudimentary, with little difference between morphometric measurements in males and females. Genistein and methoxychlor had little effect on mammary glands of female offspring. Among males, both compounds were associated with an increase in branches, terminal end buds, and lateral buds, with the effect being statistically significant for genistein at the 800 ppm dietary level. **[The Expert Panel noted that the pair-wise comparison to the 300 ppm group gave a *P* value of 0.06, using a Bonferroni correction.]** There was no interaction between genistein and methoxychlor. Histologic evaluation of tissue sections were interpreted as showing an effect of genistein exposure on lateral bud formation, and the PCNA labeling index confirmed this impression for the 800 ppm genistein group (52% cells stained in the genistein 800 ppm group compared to 35% in the control group **[estimated from graph, *P*<0.05]**). There was no significant interaction with methoxychlor exposure. IGF-1 receptor staining was described as higher in the group exposed to genistein 800 ppm **[data not shown]**. Progesterone receptor and ER staining was performed only for the control group and the group exposed to genistein 800 ppm + methoxychlor 800 ppm and was described as increased **[no quantitative data were presented]**. Serum prolactin and IGF-1 were not shown to be affected by genistein treatment. The authors added in the study Discussion section that dietary genistein did not result in an increase in uterine weight in this study. **[No information was given in the study Methods or Results section concerning the evaluation of uterine weight. This information was presented in a previous paper *(633)*.]** The authors concluded that genistein exposure enhanced the differentiation of mammary glands, expressed as an increase in lateral buds, as opposed to methoxychlor, which produced ductal proliferation.

In a continuation of this study *(662)*, 1 pup/sex/litter (n=10 litters) were weaned to their dams' diet on PND 22, and inguinal mammary glands were removed on PND 90 for evaluation in whole mount and histologic section. Trunk blood was collected for determination of 17β-estradiol, testosterone,

LH, FSH, growth hormone, IGF-1, and prolactin. RNA was extracted from mammary tissue for microarray analysis against a panel of 1176 genes implicated in cellular responses to stress and toxicity. Genistein at 300 and 800 ppm increased mammary gland size and density in male rats. Alveolar proliferation was more prominent at 800 than at 300 ppm. There were no detected genistein-related effects on serum hormone levels, although the 800 ppm dose level reduiced serum IGF-1. In the microarray analysis, there were 10 genes that were down-regulated and 23 genes that were upregulated by genistein treatment. Androgen receptor was one of the down-regulated genes and ERα was one of the up-regulated genes.

Strengths/Weaknesses: Strengths include the use of multiple dose levels, which allowed for an assessment of dose-response relationships, and the use of an exposure period that included the neonatal period. The soy- and alfalfa-free diet, the verification of homogeneity and concentrations of test diets, and the monitoring of individual dam body weights and feed consumption are additional strengths. A further strength of the study is dosing covered all potentially important critical windows of exposure for mammary gland development. The high dose of methoxychlor was not realistic; consequently, the data may not reflect the interactions of these agents at low dose levels.

The lack of culling could lead to litters of different sizes and different exposures/pup. Offspring remained with the same dams and were not cross-fostered. After weaning, rats remained on the same diet until the end of the study. Thus, it is not entirely clear how the longer observation periods are relevant to the goals of this project. The earliest time point (PND 30) is probably useful. The number of rats per cage is not clear and may not have been the same throughout the early experimental period, as the numbers were adjusted to "up to four" at weaning. At PND 55, numbers were adjusted to two same-sex littermates/cage. The animals reported are a subset of another study. It is not clear if the study was specifically designed/powered for all endpoints reported herein, or if these are secondary endpoints with respect to the original study. Rationale for low genistein dose is based on that in the NIH-07 diet; not clear if this is a relevant rationale but the exposure may be relevant to humans. The higher genistein and methoxychlor doses may not be relevant to human exposures. At PND 21, female body weights in the 800 genistein diet are significantly lower than in controls, potentially indicative of toxicity or differences in nutrient intake. Lack of effect on uterus weight at 800 ppm of genistein is inconsistent with their findings presented in previous work. The low dose and the first time point may be the most useful data in this study; the dose response data are useful only if all the exposures are relevant to humans.

Utility (Adequacy) for CERHR Evaluation Process: This study has limited utility because the authors used multiple dose levels, route of exposure, and exposure over all critical windows of development relevant to mammary gland development. The study statistically sound and the outcome measures contribute to understanding of the effect of phytoestrogens on mammary gland development. A limitation of the study for the assessment of soy infant formula is treatment during lactation as well as gestation.

3.3.3 Brain and Behavior Endpoints

A number of studies examined the effects of genistein or other isoflavone exposure on brain structure and/or behavior in rats. None were identified for mice. The studies are presented in alphabetical order by first author's last name.

Bateman and Patisaul, 2009 ***(317)***, supported by NIEHS, compared the effects in female rats of neonatal exposure to genistein by sc injection on pubertal onset, estrous cyclicity, GnRH activation, and kisspeptin (KISS) content in the anteroventral periventricular (AVPV) and arcuate (ARC) nuclei of rats. Other treatments in the study included estradiol benzoate (EB), an ERα agonist, an ERβ agonist and equol. Eight litters of Long Evans rats (Charles River, NC) were cross fostered so that each litter had 6-10 female pups. Animals were fed a phytoestrogen-free diet (AIG-93G) **[bedding not described]**. Beginning on the day of birth (PND 0), the pups (n=8-16 pups female per group) were administered subcutaneous injections once daily through PND 3 (total of four injections) of sesame oil (vehicle control; 0.05 ml), estradiol benzoate (EB; 50 μg), the ERα agonist propyl-pyrazole-triol (PPT; 1 mg/kg bw), the ERβ agonist diarylpropionitrile (DPN; 1 mg/kg bw), racemic equol (EQ; 10 mg/kg bw), or genistein (GEN; 10 mg/kg bw). All compounds were dissolved in EtOH and then sesame oil **[Purity not stated for any compound in the article, but the author provided the following information "Purity for all of our phytoestrogens is 99% or greater. Genistein is from Indofine." Personal communication, Dr. Heather Patisaul, 10/06/2009]**. The dose of GEN is similar to the total amount of soy phytoestrogens consumed daily by children fed soy infant formula and the dose of EQ was chosen to match; the doses of PPT and DPN approximated that used in previously published studies. On PND 22 the pups were weaned and examined daily for vaginal opening, monitoring of estrous cycle began two weeks later and continued for 12-13 weeks. Animals were gonadectomized (GNX) on PND 146-PND 156 and allowed to recover. After six weeks the OVX females were injected sc with 10 μg EB followed 48 h later by a sc injection of 500 μg of progesterone. Animals were killed 6-8 hours after progesterone injection. Brains were removed and processed for immunohistochemistry. One set of brain tissue was immunolabeled for GnRH and Fos, another set was immunolabeled for KISS using immunofluorescent immunohistochemistry (ICH). One rostral AVPV section and one midlevel section of the ARC were selected for quantification of KISS fiber density; the voxel counts were averaged within the substack to obtain a single measure for each section. The day of vaginal opening, percent of immunopositive GnRH cells co-labeled with Fos, and the density of voxels containing KISS fibers were compared by one-way analysis of variance (ANOVA) followed by Fisher's Least Significant Difference (LSD) post hoc test.

Vaginal opening was significantly advanced by EB (n=15) and GEN (n=8) but not EQ (n=10) compared to controls (n=12); neither PPT nor DPN affected the day of vaginal opening. Regular 4-day estrous cycles commenced in all groups, but all the EB females (n=10) stopped cycling and entered persistent estrus within 3 weeks. By 10 weeks, less than 30% of the EQ (n=8) and the GEN (n=7) treated females displayed regular estrus cycles; the remainder were either in persistent estrus or diestrus. The rate at which EQ and GEN treated animals became acyclic was very similar. The PPT treated animals were in persistent estrus by 8 weeks, however 57% of the DPN treated animals were still displaying regular cycles after 10 weeks. Hormone administration successfully induced Fos expression in the GnRH neurons of the controls (n=7); but less than 2% of GnRH neurons were co-labeled with Fos in the EB females (n=8). GnRH activation was 50% lower in the EQ animals (n=4) and 70% lower in the GEN animals (n=4). None of the GnRH neurons in the PPT animals contained Fos. Neonatal treatment with EB (n=4) resulted in a lower average AVPV KISS fiber density (64% lower); AVPV KISS density was 60% (n=5) lower in the GEN group and 25% (n=8) lower in the EQ group compared to the control group (n=8). There was no significant main effect of treatment on average KISS fiber density in the AVPV for PPT (n=3) or DPN (n=4). ARC KISS fiber density was lowest in the EB treated group (n=8, 72% lower) and also lower in the PPT group (n=6); EQ (n=7), GEN (n=5) and DPN (n=7) did not affect ARC KISS fiber density.

Authors' conclusion: Neonatal exposure to endocrine disruptors can suppress GnRH activity in adulthood and ERα may play a pivotal role in this process. The data suggest that decreased stimulation of GnRH neurons by KISS could be a mechanism by which EDCs can impair female reproductive function.

Strengths/Weaknesses: Strengths include that the study was controlled for litter effect by use of cross-fostered pups such that only two at most from each litter. Each dose group thus had pups from 10 different dams. In addition, sample sizes were adequate, multiple measures were used, mechanistic end points were measured, and positive estrogen-related controls were used (EB, ERα agonist, ERβ agonist, equol). Also, GEN was administered in a dose relevant to human exposures and there were adequate animal numbers. The most significant weaknesses are that agents were administered by sc injection. Although this allows for more precision in dosing, it complicates comparing the degree of exposure to humans who ingest isoflavones. Neither the ERα nor ERβ agonist had any effect on vaginal opening (but the ERα agonist PPT did have an effect equal to EB on GnRH/fos cells), and single doses of the agents were given.

Utility (Adequacy) for CERHR Evaluation Process: This study provides limited utility for the evaluation process due to it being well designed to provide much mechanistic information. The actions of genistein were not as pronounced but similar to EB for some the measures tested. Genistein is thought to act via ERβ, however the ERβ specific agonist was not effective in these tests. This suggests that genistein has a mixed actions on both ERs.

Csaba and Karabélyos *(664)*, supported by the National Scientific Research Fund of Hungary, examined the effects of a single neonatal sc injection of genistein **[purity not stated]** on the sexual behavior of adult male and female rats. Within 24 hours of birth, male and female Wistar rats were given a single sc dose of 20 μg genistein or 20 μg genistein + 20 μg benzpyrene in 0.066% DMSO. Controls were treated with the vehicle. **[The number of litters from which pups were obtained was not specified. Benzpyrene (not otherwise specified) was given because a previous study by these authors had shown an effect of this chemical on sexual behavior.]** Sexual behavior was examined starting at 4 months of age. On ~4 different days during a 2-week period, receptivity was assessed in 24 females/group during estrus. Sexual behavior with a receptive female was tested in 10 males/group during a 30-minute period, once a week, for 4 weeks. Data were averaged and evaluated by Student t test and chi-squared test.

Receptivity was not found to be significantly affected in females when evaluated by the Meyerson index (a binary evaluation of lordosis), but the lordosis quotient (lordosis response in 10 matings) was significantly increased by genistein treatment (~35% in controls compared to 45% in the genistein group). Genistein treatment significantly reduced sexual inactivity in male rats (50% of controls versus 32.5% of genistein-treated males inactive). The number of multiple ejaculations was increased by genistein treatment, with a 10% rate in the genistein group and no occurrences in controls. No significant effects were reported for mounting or intromission. No significant findings compared to controls were reported for males or females in the genistein + benzpyrene group. The study authors concluded that sexual activity in male and female rats is promoted by a single neonatal genistein treatment and that benzpyrene counters this effect.

Strengths/Weaknesses: In this study, the effects of estrous stage were controlled by only testing females during estrus. Sample sizes were sufficient for female rats (24/dose group) but were less

robust for male rats (only 10/group). It is not clear whether the authors controlled for litter effects in males or females or the litter was used as the unit of analysis. This study used Wistar rats from a closed breed colony (not commercially available). Only 1 dose level of genistein was used, which does not allow for evaluation of dose-response relationships, and the sc dose route is not relevant to human exposure. Purity of the genistein test material was not specified, and dose solutions were not analyzed to verify dose level, stability, or homogeneity. Dose volumes were not given. It was not clear how many experienced males or receptive females were used in this study. The increased activity in genistein-treated males may have been related to the fact that half of the control males were inactive, which seems high given that the males were co-housed with receptive females. Without some historical control data, it is difficult to put this information into context. Because this study used a single genistein exposure within 24 hours of birth, it is difficult to extrapolate these data for human exposure scenarios. While females were tested for receptivity after standard replacement hormones the males were tested with gonads intact. So if the genistein had any effect on testosterone levels that may contribute to variability in the males' sexual activity.

Utility (Adequacy) for CERHR Evaluation Process: This study is of no utility in the evaluation process.

Faber and Hughes *(665)*, in a study funded by the Duke University Medical School Research Fund, examined the effects of neonatal treatment with genistein, estradiol, diethylstilbestrol, and zearalenone on the physiology and morphology of the hypothalamus and pituitary of rats. Male and female CD rats were sc injected with genistein **[purity not specified]** daily in corn oil at 0, 100, or 1000 µg on PND 1–10. **[Pup weights were not given. Assuming pup body weights of 6 g at delivery and 15 g at PND 7, the genistein doses would have been 0, 17, and 167 mg/kg bw/day on PND 1 and 0, 7, and 67 mg/kg bw/day on PND 7. The isoflavone content of the feed was not specified.]** Rats were castrated on PND 21. On PND 42, rats received cardiac catheters and were randomly given iv saline or GnRH. Blood samples were collected before and 5, 10, 15, and 30 minutes following GnRH or saline treatment. Fifteen minutes following collection of the last blood sample, animals treated with saline received GnRH and vice versa. Serum was analyzed for LH by RIA. Data were analyzed by 1-way ANOVA, Students *t* test, Kruskal-Wallis 1-way ANOVA, or the Wilcoxon sign-rank test. Rats were killed on PND 49 and brains were fixed in formalin, sectioned, and stained with crystal violet acetate. SDN-POA volume was evaluated by an investigator blinded to treatment conditions, and data were compared by parametric analysis, confirmed by Kolmogorov-Smirnov testing. Serum LH levels in response to GnRH treatment were evaluated in 7–15 rats/sex/group.

In both males and females, treatment with 100 µg genistein significantly increased LH secretion compared to controls **[~3.5-fold in males and 2-fold in females when evaluated as AUC]**. No increase in serum LH levels was noted in rats from the 1000 µg genistein groups. LH responses in the 1000 µg genistein groups were similar to those in rats treated with ≥100 µg zearalenone. SDN-POA volumes were evaluated in 6–11 rats per sex in the control and 1000 µg genistein groups and in 3–4 rats per sex in the 100 µg genistein groups. SDN-POA volume was significantly increased in female rats from the 1000 µg genistein group. No other significant changes were noted for SDN-POA volume in rats treated with genistein, but the study authors noted that only 3 males were included in the 100 µg genistein group. SDN-POA volume effects in females from the 1000 µg genistein group were similar to those of females in the 0.1 µg diethylstilbestrol and 1000 µg zearalenone groups. The study authors concluded, "These data show that exposure to environmental estrogens early in development alters postpubertal response to GnRH and 'androgenizes' the SND-POA."

Strengths/Weaknesses: Strengths of this study include use of adequate numbers of animals, a well-defined window of exposure (PND 1–10), and comparison of results with 2 other compounds (diethylstilbestrol and zearalenone). Two doses of genistein were used. The sc route of administration has less validity to humans than oral exposure. Other weaknesses include no cross fostering and treating all members of each litter with the same compound.

Utility (Adequacy) for CERHR Evaluation Process: This study is of limited utility for the evaluation process due to use a sc injection route of administration. The study showed that a relatively low genistein dose triggered an increase LH response to a GnRH injection, while a high dose (not relevant to humans) triggered changes in SDN-POA of females (in that the females had a larger SDN volume that was no different from genistein-treated males). SDN is a morphologic marker of central nervous system differentiation and a homolog is present in human brain

Faber and Hughes *(666)*, funding source not identified, treated female CD rat pups with oil vehicle or genistein **[purity not given]** by sc injection from the day of delivery (PND 1) through PND 10. Daily genistein doses were 0 (n=9), 1 (n =5), 10 (n=6), 100 (n=9), 200 (n=5), 400 (n=9), 500 (n=6) , or 1000 (n=7) µg. **[Pup weights were not given. Assuming pup body weight of 6 g at delivery and 15 g at PND 7, genistein doses would have been 0, 0.17, 1.7, 17, 33, 67, 83, and 167 mg/kg bw/day at delivery and 0, 0.07, 0.7, 7, 13, 27, 33, and 67 mg/kg bw/day on PND 7.]** All females from each of 2 litters were represented in each dose group. Animals were ovariectomized on PND 21, and on PND 42 right atrial cannulas were placed under ketamine anesthesia. Four hours later, animals were injected through the cannulas with saline or GnRH. Blood samples were collected for measurement of LH prior to saline/GnRH, and 5, 10, 15, and 30 minutes after injection of saline/GnRH. On PND 49, animals were killed and the volume of the SDN-POA was estimated from cresyl-violet stained brain sections. LH concentrations and SDN-POA volumes were compared using ANOVA.

Basal LH concentrations were higher than the control value after treatment with 100 or 400 µg genistein. There was an increase in serum LH after GnRH in all groups except the group treated with genistein 1000 µg. The LH response to GnRH peaked at 5 or 10 minutes. There was an interaction of time from GnRH administration and genistein dose through 10 minutes. Thereafter, there was no detected relationship between LH concentration and treatment group. **[The study abstract indicates that progressive exposure to genistein was associated with a suppression of LH response to GnRH; however, data were not presented in the paper to support this point, and the data figure did not appear to support this point.]** The volume of the SDN-POA was significantly increased in the groups exposed to 500 and 1000 µg/day genistein. Unfortunately no male data are presented these would help assess the extent of masculinization. Volumes estimated from a graph in the paper are indicated in **Table 130**. **[Benchmark dose[14] calculations are BMD_{10} 258 µg/pup/day, $BMDL_{10}$ 74 µg/pup/day, $BMD_{1\ SD}$ 708 µg/pup/day, and $BMDL_{1\ SD}$ 424 µg/pup/day.]**

Strengths/Weaknesses: Strengths of this study include use of adequate numbers of animals, relevant time-frame of treatment (PND 1–10), examination of several parameters (GnRH response, SDN-POA), and the large range of genistein doses, which were lower than in a previous study. The sc route of administration is not as well related to human exposure as an oral route. An additional weakness is that litter effects were not controlled for. All female pups within a litter received the same treatment. Each dose was given to 2 litters and results are based on all the females in those litters.

[14] See the footnote to **Table 95** for an explanation of the use of benchmark dose in this report.

Table 130. Volume of the SDN-POA in PND 49 Female Rats after Exposure to Genistein (Faber and Hughes, 1993)

Daily Genistein Dose, µg/pup	*n*[a]	*Volume, $mm^3 \times 10^{-3}$ (mean ± SEM[b])*
0	9	4.2 ± 1.2
1	5	4.3 ± 1.2
10	6	5.1 ± 0.4
100	9	6.5 ± 0.8
200	5	5.9 ± 0.7
400	9	4.6 ± 0.8
500	6	7.5 ± 0.7[c]
1000	7	9.2 ± 0.8[c]

[a] Assumed from data presented for LH response to GnRH.
[b] Data were estimated from a graph by CERHR.
[c] Significantly different from control.
From Faber and Hughes, 1993 *(666)*.

Utility (Adequacy) for CERHR Evaluation Process: This study showed that low genistein doses had non-androgenic, pituitary-sensitizing effects, but higher (pharmacological) doses mimicked typical estrogen effects in masculinizing the brain. Dose-dependent differences were illustrated in this study. This study is of limited utility for the evaluation process due to use a sc injection route of administration.

Flynn et al., 2000 ***(667)***, supported by NIEHS and FDA, examined the effects of gestational and postnatal exposure to genistein on a variety of behaviors in male and female rats. Female Sprague Dawley rats were fed a soy- and alfalfa-free diet for 2 weeks prior to mating. Dietary concentrations of genistein and daidzein were below the 0.5 ppm limit of detection. Genistein (99% purity) was added to the diet beginning on GD 7 (plug=GD 0) at concentrations of 0 (n=12), 25 (n=11), 250 (n=12), or 1250 (n=12) ppm. The authors estimated that a 250 g rat would consume 20 g feed/day, giving estimated genistein intakes of 0, 2, 20, and 100 mg/kg bw/day. Litters were culled to 4 males and 4 females on PND 2 (day of birth=PND 1). Fostering of pups was used rarely to maintain litter size and distribution; most pups were reared by their own dams. Offspring were weaned on PND 22 to the same diet fed to the dam until the offspring were killed on PND 77. Animals were housed with same-sex siblings, 2 to a cage. Behavioral testing was performed as follows:

Open-field activity: One male and 1 female per litter were tested on PND 22–24, PND 43–45, and PND 65–67 (a different pair was used at each age).

Play behavior: Two males and 2 females were individually housed on PND 34. After 24 hours of isolation, animals were reunited with their same-sex sibling, and number of pins was counted over 5 minutes.

Running-wheel activity: One male and 1 female from each litter were housed individually in a cage with a running wheel on PND 63. Number of wheel revolutions by 12-hour photoperiod was counted over the next 14 days.

Taste: One male and 1 female from each litter were given access to 2 drinking water options, 1 containing regular water and the other containing either 0.03% saccharin (PND 69–71) or 3% saline (PND 73–75). Fluid intake from each bottle was measured using bottle weight and expressed as ml/kg bw/day, using the PND 70 body weight determination.

Statistical analysis was performed using ANOVA or multivariate techniques for repeated measures. Post-hoc Dunnett's test was used for comparisons with the control group.

Dam body weight was significantly decreased in the 1250 ppm group compared to the control on GD 21, and feed intake was significantly decreased in this dose group on PND 15–21 (during which time pups probably contributed to feed intake). There were no detected treatment-related effects on gestational duration, total pups/litter, live pups/litter, or sex ratio, but average weight per live pup was reduced at the 1250 ppm dose (mean ± SEM 5.86 ± 0.18 g compared to the control weight of 6.52 ± 0.18 g, $P<0.05$). **[Benchmark dose[15] calculations: BMD_{10} 1226 ppm, $BMDL_{10}$ 912 ppm, $BMD_{1\ SD}$ 1215 ppm, and $BMDL_{1\ SD}$ 844 ppm.]**. Beginning on PND 42, offspring body weight until termination at PND 77 was reduced in both males and females at the high dose. **[A benchmark dose was not calculated due to difficulty estimating the underlying data points from the figures.]**

There was no detected effect of treatment on open field or running wheel activity either in the dark or light photoperiods. The number of pins in 5 minutes showed a treatment effect using ANOVA, but there were no detected differences of any dose group from control on post-hoc testing. There was no detected treatment-related taste preference for saccharin-treated water, but saline ingestion was increased by treatment at the 1250 ppm genistein level. The authors found this effect to be consistent with the known role of perinatal estrogens in increasing adult salt consumption and postulated that the genistein exposure in this study feminized males and hyper-feminized females in this regard. They cited studies with similar effects on salt consumption after perinatal exposure to other estrogenic compounds. The lack of detected genistein effect on the other behaviors, which showed sexual dimorphism in control animals in this study or in other studies, was interpreted by the authors as possibly due to the relative weakness of genistein as an estrogen or to its primary activity at ERβ rather than the ERα. **[Some of these data were presented again by Slikker et al., 2001 *(668)*.]**

Strengths/Weaknesses: Strengths were adequate numbers of rats/group (11–12 dams/group), consideration of the dam as the experimental unit, and use of multiple behavior tests. Genistein was given to rat pups *in utero* and treatment was via mom's diet. Data on the number of grams consumed by pregnant and lactating dams were presented, although by PND 14 pups may be eating solid food and contribute to the increase in amount of food consumed. A weakness of the study is uncertainty about exposures due to administration through feed without monitoring of feed consumption. The highest dose level was not relevant to human exposure. Although how much pups are actually experiencing is not known based on dam's consumption.

Utility (Adequacy) for CERHR Evaluation Process: This study is of limited utility in the evaluation process. Because the only clear effects were seen with a dose higher than is expected for human exposures, it is suggested that typical long-term human exposures will not induce adverse behavioral effects. Overall, few measures were affected by GEN ingestion.

[15] See footnote to **Table 95** for an explanation of benchmark dose.

Levy et al., 1995 ***(669)***, supported by Duke University and the Public Health Service, examined male and female offspring following treatment of pregnant Charles River CD rats (purchased time mated) with sc genistein 25 mg/animal/day, genistein 5 mg/animal day, diethylstilbestrol 5 µg/animal/day, estradiol benzoate 50 µg/animal/day, or corn oil vehicle (n=4 animals/treatment) on GD 16–20 (or GD 15–20 for two of the diethylstilbestrol-treated animals). **[Chemical purities were not specified. Estimated maternal weight-adjusted doses were 75 and 15 mg/kg bw/day for genistein, 15 µg/kg bw/day for diethylstilbestrol, and 150 µg/kg bw/day for estradiol benzoate.]** Dams were allowed to deliver their litters (PND 1 if observed before noon). Pups were weighed and anogenital distance measured on PND 1. Pups were presumably nursed by their own dams without culling and were weaned on PND 21. Litters were divided into two groups. **[It was not stated whether whole litters were assigned to different groups or proportions of pups within each litter were assigned to different groups; it was also not stated whether the assignment to the 2 groups was random or resulted in equal numbers of litters or pups in the 2 groups.]** One group underwent castration on the day of weaning, and on PND 42, the right heart was cannulated. Four hours later, blood was collected from the cannula for determination of LH, and GnRH was administered. Blood was collected 5, 10, 15, and 30 minutes later, and LH was determined by RIA. The animals were decapitated under anesthesia, and brains were removed and blocked. Sections were taken for determination of the volume of the SDN-POA. In the second group of animals, females were separated from males and monitored for vaginal opening. After vaginal opening, daily vaginal smears were taken for a characterization of the estrous cycle until PND 90.

Statistical comparisons were made by ANOVA with post-hoc Fisher's Least Significant Difference test, except for age at vaginal opening (Kruskal-Wallis followed by Mann-Whitney *U* test) and vaginal cyclicity (compared "qualitatively"). **[Comparisons were made by treatment group without apparent regard to litter of origin.]**

Diethylstilbestrol and estradiol benzoate were said to delay parturition and increase rates of stillbirth and pup death before PND 10 **[data not shown]**. There was a decrease in the birth weight of female pups after exposure to genistein 25 mg/dam/day **[estimated from a figure as a 14% decrease in weight]**. Diethylstilbestrol and estradiol benzoate were associated with a larger decrease in birth weight in both sexes. Anogenital distance was decreased in male pups by diethylstilbestrol, estradiol benzoate, and genistein at 5 mg/dam/day, but no effect of genistein at 25 mg/dam/day was detected. The AGD were not corrected for body weight and since these were lower in the DES and E litters it is likely that the AGD differences in these groups were due to low birth weight, not less masculinization. None of the treatments had an effect on the response of LH to GnRH in females. Diethylstilbestrol and estradiol benzoate increased the volume of the SDN-POA in females but not males, and no affect of either genistein treatment on the volume of this nucleus in either sex was detected. Vaginal opening was delayed an average of 1.8 days by genistein at 5 mg/dam/day, but was not shown to be influenced by any of the other treatments. The corn oil and genistein groups were described as having estrous cycles between 3 and 5 days in length.

The authors concluded that differences in responses to genistein, diethylstilbestrol, and estradiol benzoate demonstrate that all estrogens do not share the same biologic properties. The authors could not explain the failure of the higher dose of genistein to exert the effects seen with the lower dose but indicated that there may have been kinetic issues or interactions with the corn oil vehicle. They

also indicated that phytoestrogens in the Purina Laboratory chow given to their animals may have influenced the observed effects.

Strengths/Weaknesses: One strength of the study was the well-defined exposure period (4 gestation days). Genistein results were compared with those of estradiol benzoate and diethylstilbestrol. The numbers of animals used were small but adequate. Weaknesses of the study were that the diethylstilbestrol dose was too high and the sc dose route was not as relevant to human exposure. Another weakness is that treatment groups corresponded to litters.

Utility (Adequacy) for CERHR Evaluation Process: This study has limited utility in demonstrating that genistein did not have much of an effect on sexual dimorphism. Because results were contrary to those obtained with estradiol benzoate and diethylstilbestrol, differences in mechanism of action between compounds were suggested. Lower doses of genistein did affect anogenital distance, body weight, and onset of puberty. Another finding of interest was that the lower dose had more of an effect than the higher dose of genistein suggesting that the dose response relationship may not be linear.

Patisaul et al., 2006 ***(670)***, supported by the American Chemistry Council, evaluated the effect of neonatal genistein by sc injection on the anteroventral periventricular nucleus of male and female Sprague Dawley rats. Pregnant rats (n=5) were fed a phytoestrogen-free diet (Purina 5K96) during the last week of gestation and were permitted to litter. Pups were cross-fostered among all dams so that 4 dams reared 6 females and 6 males and 1 dam reared 5 males. Pups (n=5–8/group) were randomly assigned to receive sc injections every 12 hours of 17β-estradiol 50 µg/pup/injection, genistein 250 µg/pup/injection, bisphenol A 250 µg/pup/injection, or sesame oil vehicle. The authors estimated that the twice daily dosing with 250 µg/pup was approximately equivalent to 100 mg/kg bw/day. Injections began the morning of PND 1 (delivery=PND 0). On PND 19, the pups were transcardially perfused with ice-cold saline followed by paraformaldehyde. Brains were post-fixed in 20% sucrose in paraformaldehyde, sectioned coronally, and processed for immunohistochemistry for ERα and tyrosine hydroxylase. Sections were counterstained with Nissl stain. Cells of the anteroventral periventricular nucleus positive of ERα, tyrosine hydroxylase, or both were counted. Statistical analysis used 2-way ANOVA with sex and treatment as factors followed by 1-way ANOVA and post-hoc Fisher's Least Significant Difference test.

There was a significant effect of sex on tyrosine hydroxylase-positive cells in the anteroventral periventricular nucleus with the number in males about 29% that of females **[estimated from a graph]**. The effects of treatment are summarized in **Table 131**. The authors concluded that neonatal treatment with genistein interfered with the normal testosterone-associated masculinization of the anteroventral periventricular nucleus. Because is testosterone is aromatized to 17β-estradiol in the brain, the authors interpreted this effect of genistein as anti-estrogenic.

TH is sexually dimorphic is the control animals. In females, genistein had no effect on cells but in males genistein increased the numbers of TH cells. The finding in males suggests an anti-estrogenic action of genistein. There were no differences in ER containing neurons. In females, genistein reduced the percentage of the TH cells that also expressed ER. Perhaps the early genistein treatment decreased numbers of ER selectively in TH cells. Cells staining for both ERα and tyrosine hydroxylase are not present in rodents after puberty, and the authors believed that these cells may play a role in the organization of the LH-surge. They postulated that the decrease in these cells with neonatal exposure to genistein may result in cycle disruption in adult hood.

Table 131. Effect of Neonatal Treatments on the Rat Anteroventral Periventricular Nucleus (Patisaul et al., 2006)

Endpoint	Females		Males	
	17β-estradiol	*Genistein*	*17β-estradiol*	*Genistein*
Number of cells positive for:				
Tyrosine hydroxylase	↓50%	↔	↔	↑2.1-fold
ERα	↔	↔	↔	↔
Both	↓38%	↓31%	↔	↔
Percent of cells positive for ERα + tyrosine hydroxylase	↔	↓48%	↔	↓50% (P=0.1)

↑,↓,↔ Statistically increased, decreased, or unchanged compared to within-sex sesame oil control.
P<0.05 except where noted.
Estimated from graphs in Patisaul et al., 2006 *(670)*.

Authors' conclusion: The results suggest that acute exposure to endocrine-active compounds during a critical developmental period alters AVPV development.

Strengths/Weaknesses: Strengths include use of phytoestrogen-free chow, assessment of multiple neural measures, and use of 17β-estradiol as a positive compound and litters were cross-fostered. In addition, the samples sizes were sufficient. Weaknesses include administration of genistein by sc injection, the use of only a single genistein dose level, lack of adjustment for body weight, and examination of only a small portion of postnatal development.

Utility (Adequacy) for CERHR Evaluation Process: This report is of limited utility for the CERHR evaluation process. The authors have show that genistein can act in both male and female developing brain (AVPN). It suggests a presumably anti-estrogenic action of genistein in males and also suggests a potential mechanism by which genistein could affect puberty, via ER regulation of TH cells in AVPV.

Patisaul et al., 2007 ***(671)***, supported by the American Chemistry Council, examined whether exposure of neonatal male rats to genistein or bisphenol-A by subcutaneous injection affected development of two sexually dimorphic brain regions: the anteroventral periventricular nucleus of the hypothalamus (AVPV) and the sexually dimorphic nucleus of the preoptic area (SDN). Five litters of Sprague Dawley (CRL, Raleigh, NC) rats were used on study, rats were fed a soy-free, phytoestrogen-free diet (Purina Diet 5K96) **[bedding not described]**; the day of delivery was defined as PND 0; only male pups were used in this experiment. Male pups were assigned to one of three treatment groups (n=5-8 pups/group): oil (OIL, control), genistein (GEN, 250 μg), or bisphenol-A (BIS, 250 μg) **[Purity not stated for any compound in the article, but the author provided the following information "Purity for all of our phytoestrogens is 99% or greater. Genistein is from Indofine." Personal communication, Dr. Heather Patisaul, 10/06/2009]**. Treatment was by subcutaneous injection every 12 hours beginning on PND 1 (total 4 injections); all treatment were dissolved in 10% EtOH in sesame oil. The doses were consistent with previous studies, the dose for bisphenol-A was approximately twice the oral NOAEL; genistein was administered at the same dose to allow direct comparison with bisphenol-A. On PND 85, the males were gonadectomized (GNX), age-matched females (untreated) were ovariectomized (OVX), and the endogenous hormone levels were allowed to diminish for 11 days. After 11 days of recovery, the GNX males and OVX females were subcutaneously injected

with 10 μg of estradiol benzoate (EB), followed 48 hours later by a subcutaneous injection of 500 μg of progesterone. Eight hours after the progesterone injection, all animals were killed by transcardial perfusion and the brains were removed. One series of sections was used for Nissl staining, and one series was used for immunohistochemistry. The following were quantified: volumes of the AVPV and SDN, number of calbindin-ir cells (immunoreactive for calbindin), number of GnRH-ir cells (immunoreactive for gonadotropin-releasing hormone), number of Fos-ir (immunoreactive for Fos) cells, and number of cells labeled for both GnRH and Fos. Each measure was analyzed by one-way analysis of variance (ANOVA) with treatment as the main factor; significant effects were followed up by post-hoc analysis using Fisher's Least Significant Difference test. The percentage of GnRH-ir cells immunoreactive for Fos was also calculated for each treatment group and statistically assessed by ANOVA.

The volumes of SDN and the CALB-SDN (calbindin expressing neurons) were significantly higher in the control males than the OVX females. Bisphenol-A or genistein treatment affected neither the SDN volume nor the CALB-SDN volume. Control males had significantly more calbindin-ir calls in the CALB-SDN relative to control females. The number of calbindin-ir cells in the CALB-SDN was significantly greater in the males treated neonatally with either bisphenol-A or genistein when compared to control males. AVPV volume was significantly greater in OVX females compared to control males. AVPV volume was significantly greater in males treated with genistein, but not bisphenol-A, when compared to control males, to a size statistically indistinguishable from that of OVX females. The absolute number of GnRH neurons did not differ among treatment groups; Fos expression was not induced in males regardless of treatment.

Authors' conclusion: The results suggest that acute exposure to endocrine-active compounds during a critical developmental period can independently alter nuclear volumes of sexually dimorphic nuclei and their phenotypic profiles in a region-specific manner.

Strengths/Weaknesses: The strengths are multiple measures and cross-fostering which controls for prenatal litter effects. Two endocrine active compounds were used and compared. Weaknesses include a small number of dams and litters used. Also, use of a sc route of administration rather than oral is a weakness. The number of different histological measures taken from each brain may be on the verge of too many making it hard to get well-matched sections from each rat in each condition.

Utility (Adequacy) for CERHR Evaluation Process: The study is of limited utility for the evaluation process but suggests that genistein has neural effects.

Patisaul et al., 2008 ***(672)*****,** supported by the American Chemistry Council, investigated the effects of exposure of male and female neonatal rats to genistein by subcutaneous injection on sex differences in the density of three hypothalamic sites: the ventromedial nucleus of the hypothalamus (VMNvl), the arcuate nucleus (ARC), and the dorsomedial nucleus of the hypothalamus. Rats were fed a soy-free, phytoestrogen-free diet (Purina Diet 5K96) **[bedding not described]**; the day of delivery was defined as PND 0. *Experiment #1*: Adult Sprague Dawley (CRL, Raleigh, NC) rats (10 male and 10 female) arrived on PND 75 and were killed 8 days later by transcardial perfusion without further treatment. *Experiment #2*: Five litters of Sprague Dawley (CRL, Raleigh, NC) rats were used, pups were assigned to one of three treatment groups: oil (OIL, control, n=8 males, 8 females), 17β-estradiol (E_2, 50 μg, n=8

females), or genistein (GEN, 250 μg, n=8 males, 8 females) **[Purity not stated for any compound in the article, but the author provided the following information "Purity for all of our phytoestrogens is 99% or greater. Genistein is from Indofine." Personal communication, Dr. Heather Patisaul, 10/06/2009]**. Treatment was by subcutaneous injection every 12 hours beginning on PND 1 (total 4 injections); all treatments were dissolved in 10% EtOH in sesame oil. Sprague Dawley rats; 0, 250 μg genistein by sc injection every 12h beginning on PND 1 for a total of 4 injections **[assuming a Sprague Dawley rat pup weighs 7.5 g, each dose would be 33.3 mg/kg bw/d. Two injections per day for a total of 66.7 mg/kg bw/d genistein].** The dose for 17β-estradiol has previously been shown to effectively masculinize other sexually dimorphic nuclei in the hypothalamus; the dose of genistein was sufficient to alter a number of sex differences in the anteroventral periventricular nucleus of the hypothalamus (AVPV). Pups were weaned on PND 21 and maintained on the soy-free diet until adulthood. *Both experiments*: On PND 85, the animals were gonadectomized and endogenous hormone levels were allowed to diminish for 11 days. After 11 days of recovery, the animals were subcutaneously injected with 10 μg of estradiol benzoate (EB), followed 48 hours later by a subcutaneous injection of 500 μg of progesterone. Six to eight hours after the progesterone injection, all animals were killed by transcardial perfusion and the brains were removed. One set of tissues was used for immunolabeling of 5-HT (serotonin), ERα, and HuC/D. *Experiment #1 only*: a second set of sections was used for immunolabeling of glutamic acid decarboxylase (GAD)-65, ERα, and HuC/D. 5-HT and GAD65 fiber density, HuC/D and ERα immunoreactivity in the VMNvl, and 5-HT fiber apposing HuC/D- and ERα-immunolabeled cells in the VMNvl were quantified. *Experiment #1*: Differences between groups were assessed using a t test (separate variance, Bonferroni adjusted. *Experiment #2*: Differences between groups were assessed using a two-tail, one-way ANOVA followed by Fisher's Least Significant Difference post hoc tests.

Experiment #1: 5-HT fiber density was sexually dimorphic in the adult VMNvl and ARC, but not the DMN (dorsomedial nucleus of the hypothalamus). GAD65-ir (immunoreactive to GAD65) in the adult VMNvl did not differ between the sexes. The percentage of HuC/D-ir (immunoreactive to HuC/D) neurons in the VMNvl that were colabeled for ERα was higher in females than males. The percentage of HuC/D immunolabeled cells apposed by 5-HT-ir (immunoreactive to 5-HT) fibers was higher in males than females, but the percentage of 5-HT contacts on ERα-immunolabeled cells did not differ between sexes. *Experiment #2*: Neonatal treatment with 17β-estradiol, but not genistein, masculinized 5-HT fiber density in the adult female VMNvl. Neonatal treatment did not significantly affect the colabeling of VMNvl ERα and HuC/D. No significant effect of neonatal treatment with either 17β-estradiol or genistein on 5-HT was observed in the ARC.

Authors' conclusion: The results suggest that the development of serotonergic inputs to the male VMNvl is orchestrated by neonatal estradiol exposure. The hormone-dependent organization of these 5-HT projection patterns may be an important developmental mechanism accounting for sex-specific behaviors in adulthood.

Strengths/Weaknesses: This study describes a new sex difference in the rat brain and tests the possibility that estradiol organizes the effect. The sample sizes were adequate. Genistein has little effect in this study. The study used a single, subcutaneous dose of genistein during to neonates shortly after birth.

Utility (Adequacy) for CERHR Evaluation Process: This study has limited utility for the evaluation process. The value is that this study shows that GEN does not act in the same manner as estradiol.

Patisaul et al., 2009 ***(673)***, supported by the National Institute of Environmental Health Sciences, examined whether neonatal subcutaneous injections of the ERα selective agonist propyl-pyrazoletriol (PPT) or the plastics component bisphenol-A (BPA) could affect KISS (kisspeptin) fiber density in the AVPV (anterior ventral periventricular) and arcuate (ARC) nuclei of the female rat hypothalamus and also examined whether neonatal subcutaneous injections of BPA, genistein or equol (EQ) could disrupt KISS fiber density in the AVPV or ARC of the male rat. As only the males were exposed to genistein and equol, only the male experiment is being summarized here. Timed pregnant Long Evans rats (CRL, North Carolina) were fed a semi-purified, phytoestrogen-free diet (AIN-93G, Test Diet, Richmond, IN) and allowed to deliver their litters **[bedding not described]**. Beginning on the day of birth, the male pups (n=6-11/group), received daily subcutaneous injections of test material for 4 days (PND 0 through PND 3). Male pups were treated with: vehicle (control), 50 μg/kg BPA, 10 mg/kg racemic equol (EQ), or 10 mg/kg genistein **[Purity not stated for any compound in the article, but the author provided the following information "Purity for all of our phytoestrogens is 99% or greater. Genistein is from Indofine." Personal communication, Dr. Heather Patisaul, 10/06/2009]**. All compounds were dissolved in the same vehicle (10 % ethanol and 90% sesame oil). The dose of BPA was the EPA LOAEL, the dose of genistein was similar to the total amount of soy phytoestrogens consumed by children fed soy infant formula, and the dose of EQ was chosen to match the genistein dose. Males were killed by transcardial perfusion of 4% paraformaldehyde; brains were removed, frozen, sliced into 35 mm coronal sections and divided into four sets−one set was used for this experiment. Tissues were also collected in a similar manner from untreated, age-matched, ovariectomized (OVX) females (n=7) and processed as an additional control. For each animal, sections comprising the organum vasculosum of the lamina terminalis (OVLT) through the caudal border of the AVPV were immunolabeled for KISS. One anterior AVPV section and one mid-level section of the ARC were selected to quantify the density of the KISS fibers; the remaining sections were used to visualize KISS-ir (KISS immunoreactivity). Voxel counts were averaged within the substack to obtain a single measure for each section. In the analysis of the male results, the untreated, age-matched OVX females were considered to be a treatment group. The density of voxels containing KISS fibers was compared by one-way ANOVA followed by Fisher's Least Significant Difference post hoc tests when appropriate.

Using the untreated OVX females as controls, there was a significant effect of treatment on AVPV and ARC KISS fiber density, but this was largely attributable to significantly higher levels in the untreated OVX females compared to the males regardless of treatment. AVPV KISS-ir levels were notably lower, but not significantly so, in the BPA treated males than the control males. This effect was not seen in the equol or genistein treated groups.

Authors' conclusion: The data implies that the KISS system is potentially less vulnerable to disruption by EDCs in males than females.

Strengths/Weaknesses: The strengths include use of phytoestrogen-free chow, and an adequate number of subjects, plus several controls. The weaknesses are only one dose of genistein, but this study was more focused on bisphenol A than genistein. The pups were cross fostered, but the treatment was given after the cross fostering (PN1-5) so it is not clear that was needed. Moreover the treatments appear to be given to the whole litter. Genistein was given subcutaneously which is not ideal, however in this manner the researchers know exactly what the animals received.

Utility (Adequacy) for CERHR Evaluation Process: This study is of limited utility for the evaluation. The conclusion is that the KISS system is not affected by postnatal genistein exposure in males. This agrees with the mechanism of action of genistein as an estrogen agonist. Since male pups are already exposed to estradiol endogenously at the time the additional estrogenic stimulation of genistein or equol has no additional effect.

Scallet et al., 2004 ***(674)***, supported by NIEHS, NTP, and NCTR, examined the effects of pre- and postnatal exposure to genistein in the diet on the sexually dimorphic nucleus of the hypothalamus in male and female rats. Sprague Dawley rats were fed 5K96, a feed similar to the NIH 31 feed, except that it contains casein instead of soy meal and alfalfa and corn oil instead of soy oil. The feed was reported to contain genistein 0.54 µg/g and daidzein 0.48 µg/g. From 28 days prior to mating and during gestation and lactation, 10 dams/group were fed diets containing genistein (>99% purity) at 0, 5, 100, or 500 ppm. Litters were culled to 4 males and 4 females on PND 2. On weaning, 10 male offspring/group and 5 female offspring/group from different litters were given the same diets as dams until they were killed on PND 140. Brains were removed, sectioned, and labeled with calbindin. Volume of calbindin-positive cells in the SDN-POA was measured using a 3-dimensional imaging system. Data were analyzed by 2-way ANOVA, followed by post hoc Fisher's Least Significant Difference test if significant interactions were observed. In control rats, the volume of calbindin-positive cells in the SDN-POA was higher in males versus females. Genistein treatment resulted in a significant increase in the volume of calbindin-positive cells in males from all dose groups **[~2–2.5-fold increase]**. No significant effects were observed in females.

Strengths/Weaknesses: Strengths of the study include adequate numbers of animals/group, use of several dose levels relevant to human exposure, and comparison with another estrogenic agent (*p*-nonylphenol). A weakness is that the broad time-frame of exposure, from 28 days before mating of females through PND 140 in the offspring, did not allow identification of sensitive developmental periods. The study is limited by examination of only 1 endpoint (size of sexually dimorphic nucleus by measuring calbindin-positive neurons).

Utility (Adequacy) for CERHR Evaluation Process: This study is of limited utility in the evaluation process. Increased calbindin-positive cells following life-long genistein exposure in male rats suggested an effect of genistein on brain development at doses relevant for human exposure. These data agree with other reports that used more restricted exposure to GEN,

Takagi et al., 2005 ***(675)***, supported by the Ministry of Health, Labor, and Welfare of Japan, examined the effect of perinatal genistein exposure (via the diet) on gene expression in the hypothalamic preoptic area of male and female rats. Pregnant Sprague Dawley rats were received on GD 3 (day of vaginal plug=GD 0) and fed a soy-free diet containing corn and wheat in place of soybean meal and corn oil in place of soy oil. Dams randomly assigned to groups (n=3/group) were fed the soy-free diet treated with genistein 0 or 1000 ppm (97% purity) from GD 15 to PND 10. Dose selection was based on a previous study that demonstrated reduced body weight but no effect on endocrine-related parameters in male rats following perinatal exposure to ≤1000 ppm genistein. Pups were killed on PND 10 and RNA was extracted from the hypothalamic preoptic area. An RT-PCR technique was used to measure expression of mRNA for ERα, ERβ, progesterone receptor, and steroid receptor coactivator in 6 pups/group. **[It is not certain if the authors meant 6 pups/sex/group, and the number of litters**

represented was not stated]. Data were analyzed by Bartlett test, ANOVA, Dunnett's test, Kruskal-Wallis *H* test, and/or Student *t* test. Genistein treatment had no detected effect on gene expression in the hypothalamic preoptic area of PND 10 rat offspring. In the same study, perinatal treatment of rats with 0.5 ppm ethinyl estradiol resulted in sexually dimorphic expression of ERα and progesterone receptor. The study authors concluded that genistein exerted no clear effect on gene expression in the hypothalamic preoptic area of perinatally exposed rats.

Strengths/Weaknesses: The dosing period, while limited, covered a critical period for brain sexual differentiation. According to the authors, the purity of genistein was >97%. Soy-free diet was used. Expression of target gene mRNA was normalized to 2 housekeeping genes (hypoxanthine-guanine phosphoribosyl transferase and glyceraldehyde-3-phosphate dehydrogenase), as well as input amount of total RNA. The results were consistent with the authors' previous findings with genistein in that exposure to 1000 ppm genistein using this exposure paradigm did not produced endocrine or reproductive effects in offspring of either sex. This study used a single dose level, on the high end of the spectrum, and greater than human exposure. This was done as a a follow-up study to a genistein study that used multiple dose levels *(640)*. Diets were not analytically characterized (e.g., concentration verification, stability, homogeneity). Feed consumption was not reported, so delivered dose cannot be determined. There was no adjustment to feed concentrations to account for the large increase in feed consumption that occurs during lactation. It is not clear whether the authors controlled for litter effect. Sample sizes were small (3 dams/treatment group). The authors reported using n=6/group for real-time RT-PCR; however, it was difficult to discern whether "6 pups/group" refers to one male and one female pup from each litter or 6 pups/sex/group, which would equate to 2 males and 2 females from each litter (assuming all 3 dams delivered viable litters). The number of litters was not given, nor was there any litter information provided (e.g., litter sizes, pup body weights). There was no indication that litters were culled.

Utility (Adequacy) for CERHR Evaluation Process: This study is of limited utility. The lack of effect of GEN in this study may be due to their analysis of tissue that included areas of the Medial Basal Hypothalamus outside of the SDN.

Wisniewski et al., 1960 ***(676)***, in a study supported by NIH, examined the effects of perinatal dietary genistein exposure on reproductive development and behaviors of male mice. A soy- and alfalfa-free diet supplemented with genistein **[purity not reported]** 0, 5, or 300 mg/kg diet **[ppm]** was fed to 16 randomly assigned female C57Bl/6 mice/group beginning 2 weeks prior to mating and during gestation and lactation. Genistein intakes in the low- and high-dose group were estimated by study authors at 20 and 1600–1900 mg/kg bw/day during gestation and 50–60 and 4000–4800 mg/kg bw/day during lactation. Developmental parameters examined included litter size, pup sex and body weight, and maternal behavior on PND 2. Anogenital distance was measured in males once/week on PND 2–21. Litters were culled and male offspring were weaned on PND 21. Males were weighed and examined for preputial separation beginning on PND 40. In adulthood, males were observed for sexual behavior with a sexually receptive female and aggressive behavior following introduction of an intruder male. Males were killed following completion of behavioral testing. Reproductive organs were weighed, sperm counts were determined, and plasma testosterone levels were measured by RIA. Experimental groups were comprised on 1 randomly selected male/litter (7–10/group). Data were analyzed by ANOVA, chi-squared test, and computation of z-scores.

The numbers of dams giving birth in the control, low-, and high-dose groups were 10, 7, and 8. Genistein did not affect gestation length, litter size, sex ratio, or pup weight. Maternal behavior was affected at the high dose as noted by significantly increased latency to retrieve the fourth but not the first pup. **[Details about maternal behavior testing were not provided.]** Mean ± SEM times to retrieve the fourth pup were 82.33 ± 9.9 seconds in the high-dose group and 51.00 ± 4.57 seconds in the control group. Anogenital distance was significantly reduced compared to the control group on PND 7 in both dose groups **[to ~3 mm in treatment groups compared to 4 mm in control group]** and on PND 21 in the low-dose group **[to ~6 mm in low-dose group and 7.5 mm in control]**. Body weights of males in the low-dose group were significantly lower than the control group on PND 14 **[9%]** and PND 21 **[17%]**. No significant genistein treatment effects were detected on age or body weight at preputial separation, reproductive behavior, reproductive organ weight, plasma testosterone levels, or incidence of reproductive organ masses. In 20-minute tests with an intruder male, mice in the low-dose group displayed significantly more defensive behaviors **[~4 compared to <1 in controls]**, increased duration of defensive behaviors **[~17 compared to 1 second in controls]**, and a shorter latency to initiating defensive behaviors **[~500 versus 900 seconds in controls]**. Based on *z*-scores for behaviors, it was determined that males in the low-dose group were less aggressive than control males. The findings discussed above were statistically significant at the $P<0.05$ level. **[With the exception of maternal behavior data, all quantitative data discussed above were estimated from graphs by CERHR.]** The study authors concluded that non-monotonic responses were observed for phenotypic and behavioral abnormalities induced by genistein in perinatally-exposed male mice.

Strengths/Weaknesses: The use of soy- and alfalfa-free chow is a strength of this study. Genistein was added to the chow and feed consumption was monitored, so the exact exposure to genistein could be determined. Weaknesses include the use of only 2 dose levels of genistein, examination of only males, and the failure to correct anogenital distance for body weight. It was not clear if litter was used as the experimental unit for statistical analyses. In addition, the results did not show dose-dependence.

Utility (Adequacy) for CERHR Evaluation Process: This report is of no utility in the evaluation process.

3.3.4 Other Endpoints Assessed in Rodents (Thyroid, Immune, Bone, etc.)

A number of studies examined the effects of genistein or other isoflavone exposure on other endpoints not covered by the health effect categories presented above. The studies are presented in alphabetical order by first author's last name.

Begum et al., 2006 ***(590)***, supported by the Japan Society for the Promotion of Science, investigated whether neonatal sc injection treatment augments the incidence of complex atypical hyperplasias (CAH) and carcinomas in female murine *PTEN* heterozygous (+/) mutant mice. Male m*PTEN* +/- mice (129 Ola x C56BL/6) with a deletion of exons 3-5 of m*PTEN* were mated with wild-type C56BL/6 females (Seac Yoshimoto, Fukuoka, Japan) **[feed and bedding not described]**. Diethylstilbestrol (DES), genistein and estriol (E_3) were dissolved in ethanol and corn oil **[purity not stated for any compound]**. Female pups were treated with daily subcutaneous injections of DES (1 ng/g/day), genistein (50 µg/g/day), E_3 (4 µg/g/day) or vehicle alone from the 1st to the 5th day after birth **[basis of dose selection not stated]**. Sixteen m*PTEN* +/- pups and 16 wild-type pups were assigned to each treatment group. Eight m*PTEN* +/- pups and 8 wild-type pups in each treatment groups were killed on the sixth day (day after last dose), the remaining pups were weaned at 21-22 days of age and surviving

mice were killed when they were 52 weeks old. The uterus was weighed and compared to the terminal body weight for both age groups. For the adults, one uterine horn was sectioned, stained with H&E for Histopathological evaluation, and the number of endometrial stromal cells was quantified. Total RNA was extracted from the other uterine horn and cDNA was generated. Quantification of PCR products was conducted using a fluorescence-based real-time detection method. Assay-on-Demand primers and probes were used for *Hoxa 10* and *Hoxa 11*. Means and standard errors were calculated and analyzed via protected Least Significant Difference of Fischer's test (ANOVA analysis).

Neonatal treatment with DES or E_3 significantly affected the ratio of uterine weight to terminal body weight (see **Table 132**). At 52 weeks of age, myometrial tumors were observed in one wild-type animal from the DES group, two m*PTEN* +/- animals from the genistein group and two m*PTEN* +/- animals from the E_3 group. Macroscopically, atrophic uteri were observed in both the E_3 and the DES treated mice.

Table 132. Mean Relative Uterine Weights (Begum et al., 2006)

Treatment (Age)	*% of Control Value*		
	DES	*Genistein*	*Estriol*
m*PTEN* +/– pups (6 days)	166*	134	188*
Wild-type pups (6 days)	163*	142	177*
m*PTEN* +/– adults (52 weeks)	45*	86	38*
Wild-type adults (52 weeks)	75	93	57*

DES = Diethylstilbestrol.
*Statistically significant.
Begum et al., 2006 *(590)*.

At histopathological examination, seven of the eight m*PTEN* +/- mice had multifocal CAH; age-matched wild-type mice did not develop these lesions. The incidence of CAH in m*PTEN* +/- mice was reduced by neonatal estrogenic treatment (88% of mice for control, 50% for DES, 38% for genistein, and 25% for E_3). The average number of focal CAH per uterine horn was significantly lower for all three treatments compared to the control. The incidence of adenocarcinomas in the m*PTEN* +/- mice treated neonatally with DES and E3 was significantly lower than the control group - there was no effect in the wild-type mice. Hyaline was deposited in the endometrial stroma in the m*PTEN* +/- mice with DES treatment (2/6 mice) and genistein treatment (4/8 mice) and in all wild-type mice. The endometrial stroma was atrophic in DES- and E_3-treated wild-type (1/8 and 2/8 mice, respectively) and m*PTEN* +/- mice (1/6 and 4/8). Vehicle-treated mice were unaffected. The density of the stromal cells was significantly lower in both m*PTEN* +/- and wild-type mice with DES, genistein and E_3 treatment when compared to the control. In m*PTEN* +/- mice treated neonatally with estrogenic agents, CAH were rare in the hyalized or atrophic area of the stroma, whereas in the same mice CAH developed in the areas containing sufficient stromal cells. *Hoxa* genes were highly expressed in the uteri of the wild-type mice and the m*PTEN* +/- mice from the vehicle group, but expression was significantly less for m*PTEN* +/-mice treated neonatally with estrogenic agents.

Authors' conclusion: Neonatal estrogenic exposure induced stromal atrophy and/or hyalinization accompanied by regressed expression of *Hoxa 10* and *Hoxa 11*, and exerted an inhibitory effect on *PTEN*-related tumorigenesis.

Strengths/Weaknesses: Strengths include the use of heterozygous *PTEN* mice for the assessment of treatment effects on CAH and a relevant timing (neonatal) of exposure. However, the relevance of single gene, non-conditional knockout models may be limited to inherited single gene loss/mutation in humans, which may not be common. Not clear if mutation in mouse model affects genistein PK/PD, or other endocrine factors that may interact with genistein. The use of one uterine horn for weight measures and histopathology while the other horn was reserved for gene expression studies is anotherstrength of this report. Use of two positive controls is also a strength of this study. Not clear an appropriate semi-purified diet was used and cannot exclude the possibility of highly variable other isoflavone exposures (which is likely if a standard lab chow was used). Number of events in each group is small for several endpoints, suggesting an underpowered study for some measures. Not clear relevance or basis of exposures. Any utility is probably limited to suggesting mechanism linked to PTEN loss, e.g., some endometrial cancers.

Utility (Adequacy) for CERHR Evaluation Process: This study has no utility for further evaluation.

Chang and Doerge *(242)*, from the FDA, examined the effects of *in utero*, postnatal, and adult dietary exposure to genistein on thyroid function in male and female rats. Sprague Dawley rats were fed a soy- and alfalfa-free diet containing genistein (>99% purity) at concentrations of 0, 5, 100, or 500 µg/g feed **[ppm]** during gestation and lactation. From weaning until 20 weeks of age, 6 pups/sex/group were fed diets containing the same genistein doses as their mothers. **[Based on assumed body weight (0.056 kg) and feed intake (0.0083 kg/day) of a weanling pup *(313)*, genistein intakes were estimated at ~0.75, 15, and 75 mg/kg bw/day for weanlings. Based on adult weights and feed intake (0.204 kg and 0.0200 kg/day for females; 0.267 kg and 0.0230 kg/day for males), genistein intakes in adulthood were estimated at ~0.5, 10, and 50 mg/kg bw/day.]** The study authors indicated that serum genistein levels in rats (discussed in Section 2) in the 0 and 5 ppm groups were equivalent to serum levels of humans consuming a typical Western diet. The 100 ppm concentration resulted in serum levels equivalent to individuals consuming a typical Asian diet or soy supplements. The 500 ppm level resulted in serum levels similar to those in infants fed soy formula. Genistein levels in blood and serum were measured by HPLC with electrospray MS detection. Microsomal thyroid peroxidase activity was determined by a spectrophotometric method measuring oxidation of guaiacol. Data were analyzed using 2-way ANOVA and Dunnett's test.

As noted in greater detail in **Section 2**, dose-related increases in genistein were observed in both serum and thyroid. A dose-dependent and significant reduction in thyroid peroxidase activity was observed at all dose levels, with activity in the high-dose group reduced by ~80% compared to controls. Loss of activity was significantly greater in females than in males and was consistent with higher levels of serum and thyroid genistein levels measured in females. The study authors reported that genistein had no effect on serum levels of triiodothyronine, thyroxine, and thyroid-stimulating hormone. **[Methods for analysis of thyroid hormones were not discussed and data were not shown.]** An additional range-finding study was conducted to determine if genistein effects on thyroid peroxidase activity were dependent upon stage of development, and it was found that effects were similar if genistein exposures occurred during adulthood or from GD 5 through adulthood **[data were not shown]**. A reduction in thyroid peroxidase activity was also observed in rats fed a soy-based diet containing 30 ppm genistein in glycosidic form, as discussed in greater detail in **Section 3.4**. An *in vitro* study demonstrated that thyroid peroxidase activity was inactivated by genistein at concentrations similar to those measured in thyroids of rats exposed to genistein in diet. The study authors concluded that

the remaining thyroid peroxidase activity following genistein exposure was sufficient to maintain thyroid homeostasis. The study authors suggested that consumption of isoflavones by humans could result in uptake by thyroid gland and inactivation of thyroid peroxidase.

Strengths/Weaknesses: Strengths of the study included use of soy- and alfalfa-free chow, determination of background genistein and daidzein levels in chow, use of 3 genistein doses (1, 100, and 500 mg/kg feed), and verification of genistein concentrations in chow. In addition, serum genistein levels were determined on PND 140. Finally, use of genistein dose levels that produce circulating levels representative of humans consuming a typical western diet as well as high doses is considered a strength of this paper.

Utility (Adequacy) for CERHR Evaluation Process: This study is of limited utility in determining developmental effects due to the endpoints analyzed, but data may be useful in interpreting other studies.

Csaba and Inczefi-Gonda *(677)*, supported by the National Research Fund of Hungary, examined the effects of a single neonatal sc injection of genistein on organ glucocorticoid receptor and ERs in male and female rats. Within 24 hours of birth, male and female Wistar rats (10 g bw) were given a single sc dose of 20 µg genistein **[2 mg/kg bw]** or 20 µg genistein + 20 µg benzpyrene. Controls were treated with the saline/DMSO vehicle. Animals were killed at 5 months of age, 8 days following ovariectomy for females. Glucocorticoid receptor fractions were prepared from liver and thymus, and ER fractions were prepared from uterus. Receptor-binding affinity and density were determined in each organ. For each measurement, organs were pooled from 5 animals. Four measurements were used in statistical analyses, which were conducted by the McPherson method. The only significant effect of genistein treatment was a reduction in density of liver glucocorticoid receptors in males. A significant increase in density of liver glucocorticoid receptors was observed in males and females treated with genistein + benzpyrene. Other significant effects in rats treated with genistein + benzpyrene included increased affinity of liver receptors in males and reduced affinity and density of thymus receptors in females. The study authors concluded that imprinting of the glucocorticoid and ERs was weak following a single injection of genistein. They noted that caution is required in the extrapolation of the single dose results to humans because human exposure to genistein is chronic.

Strengths/Weaknesses: Weaknesses of the study includes use of a single genistein dose (2 mg/kg bw), the sc route of administration, and lack of indication of the type of chow used and its phytoestrogen content. A further weakness is use of a single injection of genistein immediately after birth in pups.

Utility (Adequacy) for CERHR Evaluation Process: This study has no utility in determining reproductive effects due to the route and duration of exposure, and the endpoints analyzed, but data may be useful in interpreting other studies.

Dolinoy et al., 2006 *(678)*, supported by the National Institute of Health and the U.S. Department of Agriculture, examined whether dietary genistein supplementation of female mice during gestation shifted the coat color of heterozygous viable yellow agouti (A^{vy}/a) offspring toward pseudoagouti and the extent of the resulting DNA methylation. A^{vy} mice were obtained from Oak Ridge National Laboratory (Oak Ridge TN) from a colony that has been maintained with sibling mating and forced heterozygosity for the A^{vy} allele resulting in a genetically invariant background. Virgin females (8-10 weeks old) **[number not specified]** were assigned to receive either phytoestrogen-free modified AIN-

93G diet (corn oil substituted for soy oil), or phytoestrogen-free modified AIN-93G diet supplemented with 250 mg/kg diet of genistein **[purity not stated] [method of group assignment not stated]**. Diets were provided before mating and throughout pregnancy and lactation **[bedding not described].** At postnatal day 21 (PND 21), all *A^{vy}/a* offspring (15 litters/52 pups in unsupplemented group, 12 litters/44 pups in the genistein-supplemented group) were weaned to stock maintenance diet (LabDiet 5201), weighed, digitally photographed, and rated for coat-color phenotype. A single observer without knowledge of the treatment groups visually classified PND 21 *A^{vy}/a* offspring coat-color phenotype into one of 5 categories based on the proportion of brown to yellow in the fur: yellow, slightly mottled, mottled, heavily mottled, and pseudoagouti. *A^{vy}/a* offspring were weighed every 5 weeks from week 5 to week 60. For *A^{vy}/a* offspring, total DNA was isolated from PND 21 tail clips, PND 150 tail, liver, brain, and kidney tissue. For the methylation assay, sodium bisulfite modification of DNA was performed. PCR products were resolved by electrophoresis and sequenced manually. Sequencing products were resolved using polyacrylamide gel electrophoresis. Hepatic concentrations of *S*-adenosylmethionine (SAM) and *S*-adenosylhomocysteine (SAH) were measured using HPLC. Diet group comparisons of the proportion of offspring in each of the five coat-color classes were performed by chi-square analysis. Average intracisternal A particle (IAP) CpG methylation and site-specific CpG methylation were analyzed by two-tailed two-sample hypothesis testing of means. Relationships among genistein supplementation, *A^{vy}* IAP methylation, and coat color were analyzed by meditational regression analysis. Pearson's correlation coefficients and *p*-values of tissue type and age were calculated with STATA software. Body weight across coat-color phenotype was assessed by Conferring-corrected ANOVA. Diet group comparisons of the body weight were performed by chi-square analysis.

Maternal genistein did not significantly influence litter size, mean pup weight, percent survival, or sex ratio (data not shown). Maternal genistein supplementation shifted the color-coat distribution of genetically identical PND 21 *A^{vy}/a* offspring toward the pseudoagouti phenotype (P=0.0005): 50% of the genistein-supplemented offspring were classified as pseudoagouti or heavily mottled, compared with 23% of unsupplemented offspring. Only 7% of the genistein-supplemented offspring were categorized as yellow, compared with >21% of the unsupplemented offspring. Bisulfite sequencing methylation analysis of CpG sites in the cryptic promoter region of the *A^{vy}* IAP showed a statistically significant higher average percentage of cells methylated in genistein-supplemented offspring (n=44), relative to that in unsupplemented offspring (n=52). Analysis of site-specific methylation at 9 individual CpG sites revealed significantly different methylation between the unsupplemented and the genistein-supplemented diet groups at sites 4-9; the statistical significance for site 4 was an order in magnitude greater than sites 5-9. Average methylation in PND 21 tissues (n=5) was highly correlated with average methylation in PND 150 tissues derived from the ectoderm (brain and tail), mesoderm (kidney), and endoderm (liver). Body weight analysis of weaned offspring across coat-color classes showed significant differences starting at 25 weeks of age (week 25) and continuing through week 60. Week 60 pseudoagouti body weight was significantly lower when compared with each of the four other coat-color classes (**Table 133**).

A higher proportion of genistein-supplemented offspring are classified as pseudoagouti; hence, when compared with unsupplemented offspring, genistein-supplemented *A^{vy}/a* offspring at 60-weeks of age were more likely to be of normal weight (< 35g) and less likely to be obese (> 58 g). Approximately 23% of genistein-supplemented offspring were characterized as normal weight, compared with

Table 133. Body Weights Within Color Classes for 60-Week Old Mice (Dolinoy et al., 2006)

	Yellow	*Slightly Mottled*	*Mottled*	*Heavily Mottled*	*Pseudo Agouti*
Body Weight, g	54.7	59.5	56.9	54.0	35.6*
n	9	24	15	14	12

*Statistically significant.
From Dolinoy et al., 2006 *(678)*.

only 10% of unsupplemented offspring; there were no differences between male and female body weights observed. When analysis was restricted to animals within the same coat-color class, genistein supplementation was not significantly associated with body weight. No effect of dietary genistein on SAM or SAH was detected (data not shown).

Authors' conclusion: Maternal dietary genistein supplementation of mice during gestation shifted the coat color of heterozygous viable yellow agouti (A^{vy}/a) offspring toward pseudoagouti. This phenotypic change was significantly associated with greater methylation of six cytosine-guanine sites. The extent of the DNA methylation was similar in the endodermal, mesodermal, and ectodermal tissues, indicating that genistein acts during early embryonic development. The genistein-induced hypermethylation persisted into adulthood, decreasing ectopic *Agouti* expression and protecting offspring from obesity.

Strengths/Weaknesses: Strengths include that genistein was administered in diet, and the dose was comparable to a high-soy human diet. Genistein was consumed throughout gestation and during lactation. At weaning all pups were placed on soy-free diet. Several end points, litter composition, coat color, weights of pups at various ages, and methylation of the Avy allele were used. A weakness is the lack of control for litter effects however a large sample size was used. Treatment for the duration of gestation and lactation makes it difficult to determine when the modifications occur. Relationships among genistein supplementation, Avy IAP methylation, and coat color were analyzed by meditational regression analysis. Mediation is a hypothesized causal chain in which one variable affects a second variable that, in turn, affects a third variable. The number of animals in this study would not be able to determine a causal chain with any confidence.

Utility (Adequacy) for CERHR Evaluation Process: This study is of limited utility. The study highlights the non-estrogenic actions of genistein and provides proof of principle that this isoflavone can affect methylation status of specific genes. Not only is it possible that genistein acts on many more genes than Agouti but it has effects in multiple tissues. However, the methodology is still rather new and repetition is needed to confirm the results.

Dolinoy et al., 2007 ***(679)***, supported by the National Institutes of Health and by the Department of Energy, examined how the mouse fetal epigenome is affected by maternal dietary exposure to bisphenol A (BPA) alone or in combination with nutritional supplements (including genistein). Viable yellow agouti (A^{vy}) mice were obtained from a colony **[colony not specified]** that has been maintained with sibling mating and forced heterozygosity for the A^{vy} allele resulting in a genetically invariant background **[bedding not described]**. Virgin *a/a* females (8-10 weeks old) were assigned to one of four

diet groups: phytoestrogen-free modified AIN-93G diet (corn oil substituted for soy oil–Harlan Teklad, Madison WI) (n=16 litters/120 offspring); the modified AIN-93G diet supplemented with 50 mg/kg diet BPA (n=17 litters/124 offspring); the modified AIN-93G diet supplemented with 50 mg/kg diet BPA and 250 mg genistein/kg diet(n=13 litters/81 offspring); the modified AIN-93G diet supplemented with 50 mg/kg diet BPA and methyl donor compounds (4.3 mg folic acid, 0.53 mg vitamin B12, 5 g betaine, and 7.97 g choline chloride/kg diet) (n=14 litters/95 offspring); **[purity of compounds not stated][daily dose levels not stated]**. The dose level of BPA was designed to be an order of magnitude lower than the dietary–administered, maximum, nontoxic threshold in rodents (200 mg per kg of body weight per day); the dose levels of genistein and methyl donors were those previously used to study the effects of nutritional factors on the fetal epigenome. Experimental diets were provided 2 weeks before mating with A^{vy}/a males, and throughout pregnancy and lactation. On PND 22, all offspring were weighed, photographed, and rated for coat color phenotype before being killed. Tail, brain, kidney, and liver were collected for analysis from BPA-only and control offspring. Tail tissue was collected from the co-exposure offspring. A single observer visually classified PND 22 A^{vy}/a offspring coat color phenotype into one of five categories based on proportion of brown: yellow fur (<5% brown), slightly mottled (between 5 and 50% brown), mottled (~50% brown), heavily mottled (between 50 and 95% brown), and pseudoagouti (>95% brown). For all A^{vy}/a offspring and 50% of the a/a offspring, total DNA was isolated from PND 22 tail tissue, as well as the tail, brain (ectoderm), kidney (mesoderm), and liver (endoderm) of 10 BPA-only and 10 control A^{vy}/a offspring. To assess BPA's effect on the epigenome of the offspring, DNA methylation at nine CpG sites in the cryptic promoter region of the A^{vy} IAP (intracisternal A particle retrotransposon) was measured by bisulfite treatment and sequencing. Diet group comparisons of the proportion of offspring in each of the five coat color classes were performed by χ^2 analysis. Average IAP CpG methylation and site-specific CpG methylation between the control and BPA groups were analyzed by two-sample hypothesis testing of means and ANOVA and Bonferroni-corrected for multiple comparisons. Relationships among diet supplementation, A^{vy} IAP methylation, and coat color were analyzed by mediational regression analysis.

BPA Only Diet: Maternal dietary BPA did not significantly influence litter size, survival, weaning weight, genotypic ratio, or sex ratio (data not shown). In contrast, maternal BPA significantly shifted the coat color distribution of genetically identical PND 22 A^{vy}/a offspring toward the yellow coat color phenotype. Twenty-one percent of offspring developmentally exposed to BPA were classified as yellow compared with 10% of control offspring; while 9.6% of BPA offspring were classified as pseudoagouti, compared with 18.3% of control animals (see **Table 134**).

Table 134. Effect of Maternal Dietary Exposure to BPA or BPA Supplemented with Genistein or Methyl Donor Compounds on the Epigenome of the Offspring (Dolinoy et al., 2007)

% Offspring Exhibiting	*Maternal Diet*			
	AIN-93 Control Diet	*50 mg BPA/ kg Diet*	*50 mg BPA and 250 mg Genistein/ kg Diet*	*50 mg BPA/kg diet + Methyl Donor Compounds*
Yellow coat color	10%	21%	10%	12%
Pseudoagouti coat color	18.3%	9.6%	13%	13%
DNA methylation of CpG sites	39%	27%	–	–

From Dolinoy et al., 2007 *(679)*.

BPA-exposed offspring showed a significantly lower average percentage of cells methylated at the nine sites relative to that in control offspring (27% vs. 39% for the control group). Analysis of individual CpG sites also revealed significantly lower methylation in the BPA-exposed offspring at all nine sites. Regression analysis showed that, although a maternal BPA diet significantly influenced coat color, this association was markedly lower when average A^{vy} CpG methylation of sites 1–9 was included in the model, indicating that that methylation at the A^{vy} IAP principally mediates the effect of BPA exposure on A^{vy}/a coat color. Methylation levels in PND 22 tail tissues of BPA-exposed animals correlated highly to methylation levels in PND 22 tissues derived from the ectoderm (brain), mesoderm (kidney), and endoderm (liver) indicating that the A^{vy} methylation patterns resulting from BPA exposure are established before germ layer differentiation in the embryonic stem cells. <u>*Supplemented BPA Diets*</u>: Maternal supplementation with methyl donors or genistein restored the coat color distribution in BPA-exposed offspring to that observed in control litters. Ten to 13% of control, BPA-exposed/methyl donor-supplemented, or BPA-exposed/genistein-supplemented offspring were classified as yellow compared with 21% of offspring exposed to BPA alone. Furthermore, 15–18.3% of control, BPA-exposed/methyl donor-supplemented, or BPA-exposed/genistein-supplemented offspring were classified as pseudoagouti compared with only 9.6% of BPA-exposed offspring. Maternal nutritional supplementation likewise negated the BPA-induced DNA hypomethylation in the offspring. CpG methylation at the A^{vy} IAP of BPA-exposed/methyl donor-supplemented offspring was not statistically different from that of control offspring, indicating that maternal nutritional supplementation with methyl donors counteracted the hypomethylating effect of BPA. Genistein, at a level comparable with that consumed by humans with high soy diets, also counteracted the BPA-induced hypomethylation.

Authors' conclusion: Early developmental exposure to BPA can change offspring phenotype by stably altering the epigenome; interventions as subtle as maternal nutritional supplementation with methyl donors or genistein can nullify the deleterious effects of an estrogenic endocrine disruptor on the epigenome and can change the adult phenotype of the offspring.

Strengths/Weaknesses: The mode of administration of genistein is a strength. Since the dose is given in food and we do not know how much the mice are consuming, the genistein dose is not known (in the absence of toxicity a broad estimate could be made but would likely be of questionable value), so this may or may not be in the range consumed by humans. The authors only state that this is a non-toxic dose; not clear that assessment of toxicity is adequate. The diets were not analyzed for genistein stability, concentration, or homogeneity. Moreover, only one dose of genistein is given and it is only given along with BPA, not by itself; this lack of control for genistein alone is problematic. There does not appear to be any fostering in this study but that would be very difficult to do since they use four diets and the diets are given throughout the life of the offspring (starting before conception). If genistein or BPA affects maternal behavior this may have an impact on DNA methylation as well.

Utility (Adequacy) for CERHR Evaluation Process: This study has limited utility. It suggests that genistein can act as a hypermethylator (or methyl donor) when given in conjunction with BPA. This epigenetic action of genistein is important to consider and indicates that so-called phytoestrogens can have several modes of action, in addition to actions at an estrogen receptor. The primary value is its potential suggestion of one mechanism for how early life exposures may have long term effects through genetic reprogramming (DNA methylation). No clear value for assessing the physiological effects of genistein exposure on early development.

Guo et al., 2002 ***(680)***, supported by the Jeffress Memorial Trust and NIEHS, examined the effects of maternal dietary genistein treatment during gestation and lactation on the immune system of developing and adult rats. Sprague Dawley rats were fed a soy- and alfalfa-free diet. Rats were randomly placed into groups of 8 that received genistein (95% purity) through diet during the entire gestation and lactation period at doses of 0, 300, or 800 ppm. **[Based on dam body weights at necropsy and an assumed feed intake rate of 27 g/day (16% lower in the high-dose group)** ***(313)*****, genistein intake was estimated at 26 and 69 mg/kg bw/day in the low- and high-dose group.]** Groups administered 300 ppm genistein + 800 ppm methoxychlor and 800 ppm genistein + 800 ppm methoxychlor were also examined. Concentrations of compounds in diet were verified. Dams and pups were killed on PND 22 **[day of birth not defined]**. Spleens and thymuses were collected for counting of thymocytes and splenocytes. Individual cell types were counted using monoclonal antibody labeling and flow cytometry. Natural killer cell activity was also determined. The numbers of animals examined for all parameters included 3–7 dams/group and 6–8 offspring/group. Statistical analyses included Bartlett test, 1-way ANOVA, Dunnett's test *t* test, and Wilcoxon rank test.

In dams of the 800 ppm genistein group, significant reductions were observed in terminal body weight **[~10% lower than controls]** and feed intake (16% lower than controls). Absolute thymus weight of dams was significantly reduced **[~32%]** in the 800 ppm group but thymus weight relative to body weight and absolute and relative spleen weights were not shown to be affected. No effects of genistein treatment were detected on numbers of maternal thymocyte subsets; spleen natural killer cell activity and percentage and number of splenic natural killer cells were also not shown to be affected **[data not shown]**. At 300 ppm genistein, the percentage of $CD4^{-}CD8^{+}$ cells in spleen was significantly reduced by 23% compared to controls. Significant effects on spleen cells at 800 ppm included a 36% decrease in numbers of $CD4^{-}CD8^{+}$ cells, a 58% increase in percentage of $CD4^{+}CD8^{+}$ cells, and a **[39%]** decrease in numbers of spleen cells. Additional effects observed in dams treated with 800 ppm genistein + 800 ppm methoxychlor included decreases in relative thymus weight and in numbers of $CD4^{+}CD8^{-}$ thymocytes.

Terminal body weights were significantly reduced in offspring of the 800 ppm genistein group **[~14% in males and 10% in females compared to controls]**. Genistein treatment alone had no detected effect on offspring spleen or thymus weights. Absolute spleen weight was reduced in male offspring exposed to 300 ppm genistein + 800 ppm methoxychlor and 800 ppm genistein + 800 ppm methoxychlor, and absolute thymus weight was reduced in male offspring exposed to 300 ppm genistein + 800 ppm methoxychlor. **Table 135** lists results of offspring thymocyte counts that were statistically significant at one or more genistein doses. Compared to controls, percentages of $CD4^{+}CD8^{-}$ thymocytes were significantly reduced in both sexes exposed to 300 ppm genistein and males exposed to 300 and 800 ppm genistein (20–40% reduction in treated males and 35% reduction in treated females); numbers of $CD4^{+}CD8^{-}$ cells were significantly reduced by 39–61% in males of both dose groups. Additional statistically significant effects on thymocytes in females of the 800 ppm group included a 14% increase in percentages of $CD4^{+}CD8^{+}$ cells and a 79% reduction in percentages and an 82% reduction in numbers of $CD4^{-}CD8^{-}$ cells.

Table 136 outlines natural killer cell activity at each effector:target cell ratio tested. Natural killer cell activity was increased in males but reduced in females exposed to genistein. No effects of genistein treatment alone were detected on the numbers and types of splenic cells in male or female offspring.

Table 135. Significant Effects on Thymocytes of Rats following Prenatal and Lactational Exposure to Genistein (Guo et al., 2002)

Cell type	Genistein concentration in dam feed, ppm		
	0	300	800
Male Offspring			
$CD4^+CD8^-$, %	6.0 ± 0.5	4.1 ± 0.2*	3.9 ± 0.4*
$CD4^+CD8^-$, n × 10^6	51.4 ± 9.6	30.1 ± 2.4*	28.8 ± 4.1*
Female Offspring			
$CD4^+CD8^-$, %	8.3 ± 1.2	5.4 ± 0.3*	5.8 ± 0.9
$CD4^+CD8^-$, n × 10^6	63.7 ± 11.0	43.8 ± 2.1	42.9 ± 10.1
$CD4^+CD8^-$, %	74.2 ± 3.5	75.1 ± 1.9	84.7 ± 1.2*
$CD4^+CD8^-$, n × 10^6	547.6 ± 32.6	626.3 ± 50.0	605.4 ± 72.4
$CD4^+CD8^-$, %	9.4 ± 2.4	9.7 ± 1.5	2.0 ± 0.4*
$CD4^+CD8^-$, n × 10^6	76.0 ± 24.4	83.3 ± 15.1	13.7 ± 1.7*

Data expressed as mean ± SEM.
*$P \leq 0.05$ compared to control.
From Guo et al., 2002 *(680)*.

Additional effects that were observed in offspring co-exposed to methoxychlor were significantly decreased numbers of $CD4^-CD8^+$, $CD4^+CD8^+$, and total thymocytes in males treated with 300 ppm genistein + 800 ppm methoxychlor; decreased numbers of $CD4^+CD8^-$ thymocytes in females treated with both combinations of genistein + methoxychlor; and increased numbers of natural killer cells and $CD8^+$ lymphocytes in spleen of female offspring treated with 800 ppm genistein + 800 ppm methoxychlor.

The study authors concluded that genistein had immunomodulatory effects in rats that were dependent upon sex, age, and organ site, with greater effects observed in developing rats. It was noted that the

Table 136. Effects of Prenatal and Lactation Exposure to Genistein on Spleen Natural Killer Cell Activity in Rats (Guo et al., 2002)

Treatment, ppm genistein in feed	Effector:Target cells			
	12.5:1	25:1	50:1	100:1
Male Offspring				
0	< 1	< 1	1.4 ± 0.7	3.7 ± 0.9
300	1.0 ± 0.4	1.7 ± 0.7**	3.1 ± 0.6	5.4 ± 0.9
800	1.4 ± 0.3	1.7 ± 0.4**	3.9 ± 0.7*	6.7 ± 1.0
Female Offspring				
0	1.8 ± 0.4	2.8 ± 0.5	4.8 ± 1.0	9.1 ± 1.5
300	< 1**	< 1**	2.9 ± 0.5	6.2 ± 0.5
800	< 1**	< 1**	1.3 ± 0.4**	3.3 ± 0.4**

Data expressed as percentage of cell-specific lysis in mean ± SE.
*$P \leq 0.05$; **$P \leq 0.01$ compared to 0 ppm control.
From Guo et al., 2002 *(680)*.

lack of interaction between genistein and methoxychlor, which is also estrogenic, suggested that effects on the immune system involve mechanisms other than ER activation.

Strengths/Weaknesses: Although not stated in the article it is highly likely that the samples analyzed are either from the same study that evaluated the effects of gestational and lactational exposure to genistein on development described by You *et al.*, 2002 *(644)* or are from a replicate experiment. Therefore many of the strengths and weaknesses of the study design determined for You *et al.*, 2002 *(644)* are relevant to this study. Strengths of the study included use of soy- and alfalfa-free chow and verification of genistein concentrations in chow. Weaknesses were that only 2 genistein doses (300, 800 mg/kg feed) were used, and data were not analyzed on a per litter basis. In addition the number F_0 females per group evaluated was variable for the different endpoints and the reason is not specified. Body weight data was provided for 7 or 8 F_0 females/group, however, the thymocyte subset data was based on either 3 or 4 F_0 females for 3 of the 6 groups.

Utility (Adequacy) for CERHR Evaluation Process: This study is of limited utility in determining developmental effects due to the endpoints analyzed, but data may be useful in interpreting other studies.

Guo et al., 2005 ***(681)***, supported by NTP, examined the effect of developmental and adult exposure to dietary genistein on myelotoxicity in rats. Two weeks prior to mating, Sprague Dawley rat dams were switched from the standard NIH-31 diet to a soy- and alfalfa-free diet that contained casein as the protein source instead of soy and alfalfa, corn oil, and a vitamin mix adjusted for irradiation. The genistein concentration in the soy- and alfalfa-free diet was measured at 0.5 ppm **[mg/kg feed]**. Dams were assigned to treatment groups based on body weight. Starting on GD 7 (day of plug not specified) and continuing through the gestation and lactation period, 10 dams/group were given diet containing 0, 25, 250, or 1250 ppm genistein. The study authors estimated genistein doses at 0, 2, 20, and 100 mg/kg bw/day. The goal was to select a high dose that altered the reproductive system or endocrine-sensitive tissues but caused no major overt maternal or offspring toxicity. Dams were allowed to litter, and the day of birth was designated PND 1. Litters were randomly culled to 4 pups/sex/dose on PND 2. Some pups were fostered within the same treatment groups to maintain sex ratios. Pups were weaned on PND 22 and were fed the same diets as their dams until PND 64. Animals were killed for collection of bone marrow. **[The age at which animals were killed was not specified and is therefore assumed to be shortly after treatment.]** DNA synthesis in bone marrow was determined by ^{3}H-thymidine incorporation. Colony forming units (CFU) were determined following incubation of bone marrow cells with colony-stimulating factors that stimulate formation of non-lymphoid cells (granulocyte macrophage [GM]), monocytes (macrophage [MP]), and erythrocyte development and production (erythropoietin). Ten offspring/sex/dose were examined for each parameter. **[Distribution of litters was not discussed, but it is most likely that 1 pup/sex/litter was examined.]** Data were analyzed by Bartlett's test for homogeneity, ANOVA, Dunnett's *t* test, Wilcoxon Rank test, or Jonckheere's test.

Genistein treatment had no detected effect on body weight of male rats, but terminal body weights of high-dose females were reduced by 11% **[data were not shown]**. Results for myelotoxicity parameters are summarized in **Table 137**. As noted in **Table 137**, genistein treatment resulted in non-dose-related decreases in DNA synthesis, CFU-GM/10^5 cells, and CFU-MP/10^5 cells in male offspring. In female offspring, the number of recovered bone marrow cells was reduced at the high dose. A non-dose-related increase in CFU-GM/10^5 cells was observed in females from the low-dose group. The

Table 137. Myelotoxicity in Rats Exposed to Genistein During Development and Adulthood (Guo et al., 2005)

Parameter	*Genistein Dose in Diet, ppm*		
	25	*250*	*1250*
Males			
DNA synthesis	↓26%	↔	↔
CFU-GM/105 cells	↔	↓33%	↓26%
CFU-MP/105 cells	↔	↓26%	↔
Females			
Recovered bone marrow cells	↔	↔	↓41%
CFU-GM/105 cells	↑17%	↔	↔

↑, ↓, ↔ Statistically significant increase, decrease, or no change.
From Guo et al., 2005 *(681)*.

study authors noted the non-dose-related responses and speculated that genistein might be producing U-shaped responses proposed to occur with exposure to estrogenic substances. CFU/femur were also reported in the text of the study, and the study authors stated that statistically significant effects included 33–40% decreases in CFU-GM and 28–35% decreases in CFU-MP in all treated males. In high-dose female rats there was a 38% decrease in CFU-GM/femur, a 43% decrease in CFU-MP/femur, and a 42% decrease in CFU-erythropoietin/femur. **[No data were presented for CFU/femur results, and it is therefore not possible to determine if dose-response relationships occurred.]** The study authors concluded that genistein is myelotoxic and noted sex-specific and dimorphic effects. Other compounds with possible endocrine-mediating activity were examined, and the study authors concluded the most potent myelotoxic compound was genistein > methoxychlor > nonylphenol > vinclozolin in males. In females, myelotoxicity was greatest for genistein > nonylphenol > vinclozolin.

Strengths/Weaknesses: Strengths are that animals were maintained on a soy- and alfalfa-free diet, genistein was administered in the diet, a relevant route of exposure, and concentrations of genistein in the diet were confirmed analytically, dams were assigned to treatment groups based on body weight, genistein was used at multiple dose levels, and the exposure period included critical periods of development. Litters were culled on PND 2 to standardize growth rates. Weaknesses include lack of clarity on whether the authors controlled for litter effect in either their sampling methodology or statistical analyses and lack of adjustment for the increased feed consumption/kg bw that occurs shortly after weaning. With the exception of decreased number of recovered bone marrow cells in high-dose females and CFU/femur values, the parameters measured following genistein exposure did not follow traditional dose-response relationships (e.g., DNA synthesis only affected in low-dose males; there were greater decreases in CFU with GM at the middle dose than the high dose in males, whereas this value was significantly increased in females, but only at the low dose; and CFU with M was significantly decreased only in middle-dose males). The authors mentioned that the number of bone marrow cells obtained was a more variable parameter due to inherent variability in cutting the femurs and flushing the medullary cavities; thus, the measures that followed a more traditional dose-response relationship were less reliable. While low-dose effects and gender-specific myelotoxicity may be possible, there were no consistent patterns of effects in these results; thus, it would be useful to replicate this experiment.

Utility (Adequacy) for CERHR Evaluation Process: Although multiple genistein does were used and the route of administration is relevant for humans this study is of limited utility in the evaluation process because of results did not follow traditional dose-response relationships.

Guo et al., 2006 *(682)*, supported by NIH and NIEHS, evaluated the effects of dietary genistein during pre- and postnatally on immune response in male and female C57Bl/6 mice. Pregnant mice were obtained on GD 14 (plug=GD 0) and randomized to treatment groups of 4 or 5 mice/dose group. Animals received low-phytoestrogen chow (5K96, genistein and daidzein content determined to be ~0.5 ppm) to which genistein (>99% purity) was added at 0, 25, 250, or 1250 ppm **[mg/kg feed; estimated by the study authors to provide genistein 0, 2, 20, or 100 mg/kg bw/day to a 25-g mouse]**. The dams were continued on treated feed through the lactation period, and pups were weaned to their dams' feed on PND 22. On PND 42, pups were killed and spleens and thymuses were removed from 1 or 2 pups/sex/litter for evaluation. Organs were disrupted between glass slides, and cells were suspended for Coulter counting. Immune cell types were stained with specific antibodies for quantification by flow cytometry. Natural killer cell activity was evaluated using release of tracer from ^{51}Cr-labelled YAC-1 cells. Proliferation of splenocytes in response to anti-CD3 antibody was evaluated using ^{3}H-thymidine incorporation. Statistical analysis was performed using 1-way ANOVA followed by Dunnett's *t* test or non-parametric ANOVA followed by Wilcoxon rank test.

Dam body weights were increased at 250 and 1250 ppm genistein, and male pup body weights were increased at 25 and 250 ppm genistein. No effect of treatment on female pup body weight was detected. Spleen weight was increased by 250 ppm genistein in the dams and by 25 and 250 ppm in male pups. There were no detected alterations in dams or pups in relative spleen weight or in the pups in absolute or relative thymus weight. **[Thymus weight was not determined in dams.]** Immune cell results are summarized in **Table 138**. Because some effects were seen at a dietary genistein level of 25 ppm, an additional group of animals was exposed to 5 ppm genistein in the diet using the same protocol. **[The number of animals was not given. The authors estimated genistein intake at 0.4 mg/kg bw/day for a 25-g mouse eating a diet containing 5 ppm genistein.]** There were no detected effects on immune cell endpoints at this exposure level. The authors called attention to the sexuall dimorphic effects of genistein on immune endpoints, attributing this dimorphism to pup endocrine differences. The lack of detected effect of the high dose on most pup endpoints was attributed possibly to other genistein activities such as tyrosine kinase inhibition that might be present only at high intake levels. The authors concluded that genistein could increase activities of natural killer cells and T cells in male pups and showed sex-specific modulation of immune development in mice.

Strengths/Weaknesses: Strengths are that animals were maintained on a soy- and alfalfa-free diet, background concentrations of genistein and daidzein in the control diet were determined, genistein of high purity was administered in the diet, the route of exposure was relevant, genistein concentrations in the diet were confirmed analytically, and test diets were analyzed for stability. In an effort to control for litter effect, 1 or 2 mice per sex from each litter were randomly selected for evaluation. Genistein was used at multiple dose levels, which allows for an evaluation of dose-response relationships, and the exposure period included critical periods of development. Statistical analyses were appropriate. Weaknesses are that the day of birth was not specified as PND 0 or 1, the exposure estimates for the animals, which covered weaning through PND 42, were not well founded, and there were no data on maternal effects during gestation, litter parameters, or pup body weights prior to termination.

Table 138. Effect of Dietary Genistein on Immune Cell Endpoints in Mice (Guo et al., 2006)

Cell type	Genistein level in diet (ppm)								
	25	250	1250	25	250	1250	25	250	1250
	Dams			Male Pups			Female Pups		
Splenocytes									
$CD4^+CD8^-$									
Number	↔	↑1.7-fold	↔	↔			↔	↔	↔
Percent	↔	↓32%	↔	↔			1.2-fold	↔	↔
$CD4^-CD8^+$									
Number	↔	↑1.7-fold	↔	↑1.5-fold			↔	↔	↔
Percent	↔	↓33%	↓12%	↔			↔	↔	↔
$CD4^+CD8^+$									
Number	↔	↑2-fold	↔	↑1.4-fold			↔	↑1.7-fold	↔
Percent	↔	↔	↓31%	↑1.4-fold			↔	↑1.6-fold	↑1.4-fold
Splenocytes									
Number	↔	↑2.5-fold	↔	↑1.3-fold			↔	↔	↔
Proliferation									
Basal	↔	↑2.1-fold	↔	↔			↔	↔	↔
Stimulated	↔	↔	↔	**[↑1.4-fold]**[b]			↔	↔	↔
Natural Killer Cells									
Number	↔	↑3.6-fold	↔	↑1.7-fold			↔	↔	↔
Percent	↔	↔	↔	↑1.2-fold			↓30%	↓19%	↓30%
Activity[a]	↔	**[↑1.6–2-fold]**[b]	↔	**[↑1.5–1.8-fold]**[b]			**[↓36–62%]**[b]	↔	↔
Thymocytes									
Number	↔	↔	↔	↑1.7-fold	↔		↔	↓28%	↔
$CD4^+CD8^-$									
Number	↔	↔	↔	↑1.5-fold	↔	↔	↔	↔	↔
Percent	↔	↔	↔	↔	↔	↔	↔	↔	↔

Table 138 (continued)

Cell type	Genistein level in diet (ppm)								
	25	250	1250	25	250	1250	25	250	1250
	Dams			Male Pups			Female Pups		
CD4−CD8+									
Number	↔	↔	↔	↑1.5-fold	↔	↔	↔	↔	↔
Percent	↔	↔	↔	↔	↔	↔	↔	↑1.2-fold	↔
CD4+CD8+									
Number	↔	↔	↔	↔	↔	↔	↔	↓31%	↔
Percent	↔	↔	↔	↑1.2-fold	↔	↑1.3-fold	↔	↓5%	↔
CD4−CD8−									
Number	↔	↔	↔	↔	↔	↔	↔	↔	↔
Percent	↔	↔	↔	↓45%	↓36%	↓50%	↑1.3-fold	↑1.4-fold	↔
CD44+CD25−									
Number				↑1.5-fold	↔	↔	↔	↓42%	↓44%
Percent				↔	↔	↔	↔	↓18%	↓25%
CD44+CD25+									
Number				↔	↔	↔	↔	↓62%	↓75%
Percent				↓42%	↓35%	↓44%	↔	↔	↓64%
CD44−CD25+									
Number				↑1.3-fold	↑1.4-fold	↔	↔	↓34%	↓41%
Percent				↔	↔	↔	↔	↔	↔

↑,↓,↔ Statistically significant increase, decrease, or no change compared to 0 ppm control.
[a]Range based on different effector:target cell ratios.
[b]Estimated from a graph.
From Guo et al., 2006 *(682)*.

In some cases, sample sizes were small and variability in some parameters was difficult to explain. Many of the parameters measured following genistein exposure do not follow traditional dose-response relationships. There was little consistency between effects on cell subpopulations in the spleen and thymus. In comparison with the earlier paper in rats *(680)*, there were some cross-species (rat versus mouse) differences in the immune response with perinatal genistein exposure.

Utility (Adequacy) for CERHR Evaluation Process: While low-dose effects and gender-specific immune effects may be possible, the lack of a clear pattern of effects across these studies make these data difficult to use. This paper is of limited utility in the evaluation process.

Kaludjerovic and Ward *(683)*, supported by the International Life Sciences Institute North America, examined whether neonatal subcutaneous injections of a combination of daidzein and genistein results in greater benefits to bones of the adult mouse than either treatment alone. Outbred CD-1 mice (Charles River) were placed on a control diet (AIN93G) that was devoid of any estrogenic compounds; mice were mated and allowed to deliver **[bedding not described]**. Litters were randomized in to 1 of 5 groups: corn oil (CON, vehicle control), daidzein (DAI, 2 mg/kg bw/day), genistein (GEN, 5 mg/kg bw/day), daidzein plus genistein (DAI+GEN, 2 mg daidzein + 5 mg genistein/kg/day), or diethylstilbestrol (DES, positive control, 2 mg/kg bw/day) **[purity of the substances was not stated]**; daidzein, genistein and diethylstilbestrol were solubilized in dimethyl sulfoxide and suspended in corn oil. Treatments were administered each morning from PND 1 to PND 5 (5 doses) via a single subcutaneous injection. The doses of daidzein and genistein were selected to resemble the quantity and ratio of each isoflavone in soy protein-based infant formula. 1.5 hours after the last dose on PND 5, a subset of pups (n=4-6/group) was killed to verify the serum levels of daidzein, genistein and equol (daidzein metabolite). Body weights were measured weekly. Remaining mice were killed at 4 months of age. Femurs and lumbar vertebrae (LV) were collected and the following endpoints were measured: bone mineral content (BMC) and bone mineral density (BMD) of the left femur and LV1-LV3, biomechanical strength of the femur and LV2, microarchitecture of the femur and LV4. One-way ANOVA was performed to determine the significance of differences in microarchitecture; two-way ANOVA was conducted for all other outcomes with gender and treatment as the main effects. Student-Newman Keuls test was used for comparisons of multiple means when statistical differences were observed.

Body weights were unaffected by treatment. On PND 5, serum isoflavone concentrations of mice treated with genistein and daidzein were higher than all other groups. Females had higher vertebral BMC and BMD than males. All females groups receiving isoflavones or diethylstilbestrol had higher vertebral BMC and BMD than the female control group; Males treated with daidzein or genistein had a higher BMC of LV1-LV3 than males treated with diethylstilbestrol. BMD of LV1-LV3 was lower in diethylstilbestrol-treated males compared to all other groups. Peak load of LV2 was higher among females compared with males; there was a greater gender X treatment effect with daidzein and diethylstilbestrol females having greater vertebral peak load compared to all other groups. Femur BMC was higher among males compared to females and there was an overall treatment effect due to daidzein, as BMC was higher with daidzein compared with the control and diethylstilbestrol groups. Daidzein treatment resulted in a greater femur BMD compared with all other groups, daidzein males also had higher BMD than diethylstilbestrol-treated males. Females treated with daidzein or diethylstilbestrol and males treated with daidzein or daidzein+genistein had greater femur mid-point peak load than the female control or male diethylstilbestrol groups; all isoflavone groups and

diethylstilbestrol had a greater ultimate stiffness at femur midpoint than the control group. Females treated with daidzein, genistein and diethylstilbestrol had higher BV/TV (bone volume/total volume) at the LV4 compared to the control group, and daidzein+genistein had intermediate effects. Treatment with daidzein also resulted in higher BS/BV (bone surface area/bone volume) at the LV4 compared with all other treatment groups. Tb.Th (trabecular thickness) was higher among females treated with genistein and daidzein+genistein compared to all other treatment groups. Treatment with daidzein or diethylstilbestrol resulted in a higher Tb.N (trabecular number) at the LV4 compared to the control group, and daidzein+genistein had intermediate effects. All treatment groups had lower Tb.Sp (trabecular separation) at the LV4 than the control group, and female mice treated with daidzein or diethylstilbestrol had lower Tb.Sp than genistein- or daidzein+genistein-treated mice. At the femur neck, Tb.N was higher among diethylstilbestrol-treated females compared to the other treatment groups; females treated with daidzein and genistein had a higher Tb.Sp compared with the diethylstilbestrol group, and daidzein+genistein had intermediate effects. The outer cortical parameter was higher among females treated with daidzein, genistein, and diethylstilbestrol compared to the control group, and daidzein+genistein had intermediate effects. Treatment with daidzein and genistein resulted in higher cortical area at the femur neck compared with the control group and diethylstilbestrol, and daidzein+genistein had intermediate effects. Bone marrow area was higher among control females or treated with genistein compared with diethylstilbestrol; daidzein alone or with genistein had intermediate effects. At the femur midpoint, females treated with daidzein and genistein alone had a higher BV/TV compared with the control group, and daidzein+genistein had intermediate effects. BS/BV was higher among females treated with diethylstilbestrol compared with daidzein, with all other treatment groups having intermediate effects. Treatment with daidzein resulted in higher Tb.Th, higher cortical thickness, and greater cortical area at the femur midpoint compared with the control group or diethylstilbestrol; genistein alone or in combination with daidzein had intermediate effects. Qualitative assessments revealed that females treated with daidzein or diethylstilbestrol had improved trabecular network at the LV, with genistein and daidzein+genistein having intermediary effects. At the femur neck, females treated with daidzein exhibited visibly greater cortical thickness than all other treatment groups and the female diethylstilbestrol group had visibly improved cortical thickness compared with the control group. At the femur midpoint, the thickness of the cortical wall was greater among females treated with daidzein compared with the female control group.

Authors' conclusion: Neonatal exposure to daidzein and/or genistein had a positive effect on the skeleton of female mice at adulthood, but compared with individual treatments, daidzein+genistein did not have a greater benefit to bone in females or males.

Strengths/Weaknesses: Strengths of the study include the measurement of daidzein, genistein, and equol in serum, and it included a thorough assessment of various endpoints of bone growth. Mechanical strength of the bone tracked with BMD and BMC indicating that mechanically competent bone was made. Weaknesses include the subcutaneous route of exposure, and a short period of exposure as compared to humans since the animals were dosed on postnatal days 1 to 5, and lack of labeling and histomorphometry on bone sections to assess effects on formation versus resorption of bone.

Utility (Adequacy) for CERHR Evaluation Process: This study has no utility for the evaluation since a subcutaneous route of exposure was used although this is partially compensated by measurement of isoflavones in serum.

Klein et al., 2002 ***(684)***, supported by NIH and the National Aeronautics and Space Administration, evaluated the effects of pregnancy and lactation dietary genistein treatment on the immune system of male Long Evans rats. Adult female rats were placed on a soy- and alfalfa-free diet to which genistein **[purity not specified]** was added at 0, 5, or 300 mg/kg feed. After 2 weeks, the females were bred to males on an unspecified diet and were maintained on their assigned diets through weaning. Based on measured feed consumption, the authors estimated mean genistein intake in the supplemented groups at 0.42 and 25 mg/kg bw/day, stated to be equivalent to human isoflavone intakes on Western and Asian diets, respectively. Males were weaned on PND 21 and housed 3/cage. Half the genistein-exposed males were weaned to their dam's diet and half were weaned to the soy- and alfalfa-free diet. **[Because there were no significant differences by diet at weaning, these groups were collapsed for analysis and evaluated only by the diet to which the dam was assigned.]** On PND 70, blood was collected for measurement of plasma testosterone and thymuses and spleens were harvested. Lymphocytes were collected from both tissues and counted by CD4 and CD8 status. Splenic B cells were counted using a CD45R marker. Lymphocytes cultured with concanavalin A were evaluated for production of interleukin-4 and interferon-γ. Data analysis was performed using ANOVA with post-hoc Tukey test or Pearson product-moment analysis for correlations.

There were no detected effects of maternal diet on adult body weight or relative spleen weight. Relative thymus weight was increased 25% **[estimated from a graph]** in the high-dose genistein group. There was no detected effect of diet on splenic B cell number. Effects on T cell populations are shown in **Table 139**. There were no observed significant diet effects on production of interleukin-4 or interferon-γ by cultured lymphocytes. Plasma testosterone levels were 45–52% lower **[estimated from a graph]** in animals exposed to genistein, without an apparent dose-related effect. Plasma testosterone was negatively correlated with thymus $CD4^+CD8^+$ cell count and positively correlated with thymus $CD4^+CD8^-$ and $CD4^-CD8^-$ cell counts. The authors concluded that genistein may augment cellular immunity through a reduction in testosterone, which has immunosuppressant effects.

Table 139. T Cell Counts in Male Rats Exposed Through the Dam to Genistein in the Diet (Klein et al., 2002)

Cell Type	*Genistein Exposure Level, mg/kg feed (mg/kg bw/day)*	
	5 (0.42)	*300 (25)*
Spleen		
CD4+	↔	↔
CD8+	↔	↑1.2-fold
Total T cells	↑1.1-fold	↑1.2-fold
Thymus		
CD4−CD8−	↓59%	↓61%
CD4+CD8−	↔	↔
CD4−CD8+	↔	↔
CD4+CD8+	↑1.1-fold	↑1.1-fold

↑,↓,↔ Significant increase, decrease, or no change compared to control diet.
From Klein et al., 2002 *(684)*.

Strengths/Weaknesses: Strengths are that the dosing period covered *in utero*, postnatal, and adult stages, genistein was administered in a soy-free diet, and the dose levels did not alter the number of pups per litter at birth, sex ratio, pup birth weights, or adult body weights of the male offspring. The use of 2 dose levels is a strength in permitting evaluation of dose-response relationships but is less than ideal. Sample sizes were small (only 4 litters per treatment group) with each litter contributing 1–3 pups for sample collection; thus, there was limited control for litter effects. Other weaknesses were the use of only male offspring, the lack of indication of the purity of the genistein, the lack of information on how dams were assigned to treatment groups, the lack of analytic characterization of diets, and the lack of determination of feed consumption against actual measured body weight. Given the variance in maternal feed consumption during gestation and lactation and pup feed consumption post-weaning, it seems unlikely that the dose estimates adequately reflected genistein dose levels over the exposure period. There were no data presented on maternal or pup body weights during the dosing period. Litters were not culled until day 21, which likely resulted in differences in offspring weight and nutrition during the lactational period. In many cases, the results did not exhibit dose-response relationships, which is unusual given the 60-fold difference in dose levels.

Utility (Adequacy) for CERHR Evaluation Process: This study has limited utility for the evaluation process owing to the limitations from the use of only two dose levels of genistein in the diet and the absences of expected dose-response relationships.

Piekarz and Ward *(685)*, [support not indicated], examined whether the exposure of male and female mice to genistein (GEN) by sc injection during the first 5 days of life resulted in a higher bone mineral density (BMD) and greater biomechanical bone strength properties than mice not exposed to genistein. Ten timed-mated CD-1 mice (CRL, Quebec) were fed a control diet (AIN93G) that is devoid of isoflavones including genistein and allowed to deliver their pups **[bedding not described]**. At birth, pups were randomized into one of three groups: control (CON, vehicle), diethylstilbesterol (DES **[purity not stated]**), or genistein (GEN **[purity not stated]**). Sex distribution was not equal across groups (n=7m:11f for CON, n=14m:4f for DES, and n=12m:10f for GEN). The control group pups received the vehicle (Corn oil); the DES group pups received 2 µg of DES/pup/day as a positive control; the GEN group pups received 4 µg GEN/pup/day. The dose of DES has been shown to favorably modify bone metabolism; the dose of GEN is equivalent to the average exposure of infants to isoflavones in soy-based infant formula. Pups were injected subcutaneously with a total volume of 0.1 ml/pup/day on postnatal days 1 through 5. At 21 days of age, pups were weaned and continued on the AIN93G diet. Body weight was measured once weekly. At 4 months of age, mice were killed and blood and femurs were collected; the testes, uterus and ovaries were weighed. Serum osteocalcin and serum collagen crosslinks were measured using enzyme-linked immunoassay. Bone mineral content (BMC) and BMD of the left femur and lumbar vertebrae 1-4 (LV1-LV4) were determined by dual energy x-ray absorptiometry (DEXA). The biomechanical strength properties of the femur and LV3 were measured by three-point bending and compression testing, respectively. Two-way ANOVA was performed for most outcomes with gender and treatment as the main effects; interaction effects (gender X treatment) were assessed. One-way ANOVA was used to analyze organ weights. Student-Newman Keul's test was used for comparison of multiple means when statistical significance was observed. Difference were considered significant at $P<0.05$.

There were no effects of treatment on body weight or reproductive organ weights. Pups treated with DES and GEN had greater femur weights than the control mice. Femur length and depth did not differ between groups; but treatment with GEN resulted in greater femur width than the control or DES group. Females treated with DES had greater femur BMC than CON females and males treated with DES; males treated with GEN had a higher femur BMC than males treated with DES or control females. DES and GEN treated males had a higher femur BMD than controls. Females that received DES and GEN, and males treated with GEN had a higher femur BMD than female controls or males treated with DES. Females treated with DES and males treated with GEN had a higher femur peak load than female controls or males treated with DES. GEN and DES treatment resulted in higher BMC and BMD of LV1-LV4. Male and females treated with GEN had a higher BMD than all other groups except females treated with DES. Males and females treated with GEN and females treated with DES had a higher peak load than all other groups. There was a significant interaction of gender X treatment for both serum osteocalcin and collagen crosslinks.

Authors' conclusion: Short-term exposure to genistein during the first 5 days of life had positive effects on the femur and the lumbar spine of females, likely due to estrogenic effects, while only the lumbar spine of males benefitted from early exposure to genistein.

Strengths/Weaknesses: Strengths include the use of phytoestrogen-free diet and appropriate endpoints for the assessment of bone mineral density and strength. Weaknesses of the study include the use of the subcutaneous route of exposure, exposure was limited to the neonatal period (first 5 days), the composition of the diet was not detailed, no dose-response was assessed, circulating levels of genistein were not measured, the number of pups evaluated in the different groups was very variable. Also, DES had variable effects on bone measures, thus it may not have been the most suitable for use as a positive control.

Utility (Adequacy) for CERHR Evaluation Process: The study has no utility for the evaluation.

Ward and Piekarz *(686)*, supported by ILSI N.A.,examined whether *in utero* exposure to genistein and/or daidzein via sc injection to the dam has a positive effect on bone health, resulting in a higher bone mineral density (BMD) and greater resistance to fracture in adulthood. Male and female CD1 mice (CRL, Quebec) were adapted to control diet devoid of phytoestrogens (AIN93G) for 2 weeks and subsequently mated. On gestation day 0 (GD 0), the females were randomized into four study groups **[group size not stated]**: vehicle (corn oil) control, 3.75 mg genistein **[purity not stated],** 3.75 mg daidzein **[purity not stated],** and genistein + daidzein (3.75 mg each). Mated females received daily subcutaneous injections from GD 9 until GD 21. Doses of isoflavones were based on the level of isoflavones in a soy protein-based rodent diet in which ~ 500 mg of isoflavones are present per kg of diet. **[bedding not described]** At birth, pups remained with their mothers until they were weaned at postnatal day 21 (PND 21). On PND 21, 12 pups/sex/group were selected for continued observation **[number of litters not stated]**. Body weights were measured once weekly. On PND 120, the F_1 animals were killed, the testes, seminal vesicles, prostates, ovaries and uteri were weighed, the femurs and lumbar vertebrae (LV1-LV4) were collected; blood was collected and analyzed for serum osteocalcin, and serum collagen crosslinks were measured. Bone mineral content (BMC) and bone mineral density (BMD) were determined by DEXA; three point bending was used to determine the structural properties of the right femurs; the height, depth, width, and peak load of LV4 were determined. Two-way ANOVA was performed for all outcomes except organ weights which

were analyzed by one-way ANOVA. For both one-way and two-way ANOVA, Student-Newman Keuls's test was used for comparison of multiple means when statistical differences were observed. To determine the relationship between whole femur BMC and mechanical strength properties, linear regression analyses were performed. Differences were considered significant at $P<0.05$.

There were no differences in pregnancy outcomes between treatment groups. Weaning weight of control and genistein groups were significantly greater than the genistein+daidzein group. Final body weight and total weight gain showed no differences between groups. The reproductive organ weights were not affected by treatment. The femur weight and depth of the daidzein group was higher than the genistein+daidzein group; length and width of the femurs did not differ between groups. Females in the genistein group had higher yield load than female controls and genistein group males. Femur peak load did not differ between groups. Genistein and daidzein groups had significantly higher ultimate stiffness than the control group. The height of LV4 was significantly greater in the genistein group compared to the daidzein group, the weight of LV4 was higher in the control than the genistein+daidzein group, and the width was greater in the control than all other groups; depth did not differ between groups. The control had a higher BMC of LV1-LV4 compared to the daidzein and the genistein+daidzein groups. BMD of LV1-LV4 was higher in the control and genistein groups compared to the daidzein group; the females in the daidzein group had a lower BMD than the control females and the genistein group females. Peak load of LV4 did not differ between groups. No effect of treatment was seen on osteocalcin or on serum collagen crosslinks.

Authors' conclusion: in utero exposure to individual isoflavones, genistein or daidzein, or their combination did not confer a positive, long-lasting effect on bone health as BMD and biomechanical strength properties were not greater than control group at 4 months of age, representing young adulthood.

Strengths/Weaknesses: Strengths include the use of phytoestrogen-free diet, and appropriate endpoints for the assessment of bone mineral density and strength. Weaknesses of the study include the use of the subcutaneous route of exposure, exposure was limited to gestation, the composition of the diet was not detailed, no dose-response was assessed, the groups sizes for the F_0 females and the method for selection of the F_1 pups was not specified, and circulating levels of genistein and daidzein were not measured. The study does not appear to have been controlled for litter effect.

Utility (Adequacy) for CERHR Evaluation Process: The study has no utility for the evaluation due to use of the subcutaneous route of exposure and methodological and/or reporting short-comings.

Xiao et al., 2007 ***(687)***, supported by the US Department of Agriculture, examined the effects of dietary soy protein isolate (SPI) or genistein (GEN - soy isoflavone) during pregnancy on development of colon cancer in male progeny. Pregnant Sprague Dawley rats were received from CRL: Wilmington, MA and housed on corncob bedding. On gestation day 4 they were randomly assigned to one of three isocaloric, isonitrogenous (AIN) 93G diets containing casein (CAS 200g/kg diet), soy protein isolate (SPI 200 g/kg diet) or CAS supplemented with genistein (GEN 2.5 g/kg diet. **[purity of substances not stated; number of dams per group not stated; basis of dose selection not described; assignment of animals to study groups not described; bedding not described.]** Immediately after parturition, the dams and litters were switched to the CAS diet (represented as the CAS Group, the SPI/CAS

Group, and the GEN/CAS group), except for one subgroup of the SPI dams/litters which continued on the SPI diet (the SPI Group). At gestation day 19, amniotic fluid was collected and pooled for all fetuses of one dam on each diet; total isoflavones were extracted and quantification was performed on LC-MS. On postnatal day 2 (PND 2), each litter was culled to 5 males and 5 females (females were used on a different study). F_1 males were injected subcutaneously with the intestinal carcinogen azomymethane (AOM) in saline (15 mg/kg body weight) on PND 47 and PND 55 (n=60, 54, 59, and 45 for the CAS, SPI, SPI/CAS, and GEN/CAS groups, respectively). 20 weeks after the second AOM injection, the animals were killed and subjected to tumor evaluation, serum samples were prepared and colon mucosa was harvested. At the end of the study, serum insulin, leptin, estradiol, testosterone and IGF-binding proteins (IGFBP-1, -2 and -3) were measured. Total RNA was extracted from colon mucosa; one μg was converted to corresponding cDNA, real-time RT-PCR products were detected and cyclophilin A mRNA was used to normalize the real-time RT-PCR results. The Fisher's exact test was used to examine diet effects on tumor incidence and pathology. For comparisons of mean values between treatments, *t* test or one-way ANOVA with the Holm-Sidak method to adjust for multiple comparisons was used. Statistical significance was set at $P \leq 0.05$, a tendency for an affect was indicated by $0.05 \leq P \leq 0.01$.

The amount of GEN fed resulted in amniotic fluid levels of free and conjugated GEN comparable to the SPI diet; the SPI diet resulted in detectable levels of daidzein and its metabolites O-DMA and Equol. Similar body weights were observed for the F_1 pups at PND 2; body weights were significantly higher in the SPI/GEN group on PND 11 and 18. Body weights were lower in the CAS and SPI/CAS groups from PND 18 until PND 149. The SPI group had a significantly lower overall incidence of colon tumors compared to the CAS group; the SPI/CAS group and the GEN/CAS group had a similar tumor incidence compared to the CAS group. There was no difference in the relative ratio of single to multiple-tumor bearing animals between the SPI and CAS groups; the SPI/CAS group had a higher relative percentage of animals with multiple tumors when compared to the CAS and SPI groups; diet did not affect the average tumor weight. The percentage of adenocarcinomas was lower (not statistically significant) in the SPI, SPI/CAS and GEN/CAS groups when compared to the CAS group. Serum concentrations of insulin and leptin were significantly lower in the SPI group compared to the other groups. Serum estrogen was not affected by diet; serum testosterone was significantly lower in the SPI/CAS group, but not the SPI group. Circulating IGF-I concentrations were higher in the SPI/CAS group; IGFBP-1 and IGFBP-3 were higher in the SPI, SPI/CAS and GEN/CAS groups compared to the CAS group, there was no effect in IGFPB-2. Colonic *Igfr1* mRNAs tended to be more abundant in SPI/CAS animals. RNA transcripts encoding GLUT1; SLC2A1 exhibited a tendency to be higher in SPI/CAS animals. The colons of SPI rats had a relatively high frequency of lymphoid nodules (25% vs. 11%, 3% and 2% for the GEN/CAS, SPI/CAS and CAS groups respectively).

Authors' conclusion: Dietary exposure to a soy protein-based diet during pregnancy followed by a switch to CAS at delivery increased colon tumor multiplicity in male progeny as later adults, and also permanently altered several endocrine parameters previously linked to colon carcinogenesis. Thus, dietary protein type during pregnancy effected colon tumor multiplicity and colon tissue gene expression, as well as serum IGF-I and testosterone in the progeny of rats as later adults.

Strengths/Weaknesses: Dietary route of administration is a strength of the present report although it was difficult to determine why these exposures were selected. Source of genistein provided (Sigma);

purity is not provided but it is reasonable to assume ≥98% (per Sigma). Appropriate semi-purified AIN-based diets and adjusted for nitrogen content. Diets were prepared commercially; but it is not clear if analyzed for stability, concentration, or homogeneity. Other strengths are that animals were randomly assigned to each group and the collection of amniotic fluid on GD 19 and measurement of isoflavones by LC-MS in pooled amniotic fluid for all pups. CAS-Gen group data difficult to evaluated without a GEN-GEN control (comparable to the other postpartum diets). Potentially useful comparisons are SPI to CAS. Isoflavone composition of the soy protein isolate (SPI) group not clear, making an assessment of the exposure difficult to assess. However, while the SPI source is the same as other papers from this research group it seems unlikely that the batch is the same and no reliable assumptions about the exposures can be made. Inclusion of measures for serum insulin, leptin, estradiol, testosterone and IGF-binding proteins (IGFBP-1, -2 and -3), hormones thought to be important in colon tumor development was also a strength. The changes in hormone and adipokine levels reported may be useful in identifying mechanisms and/or suggesting areas for further investigation. Measurement of RNA transcripts for genes thought to be important in colon tumor development is another strength. The use of a single concentration is a weakness of the present report. Chemical induction of tumors, although an appropriate animal model, has limited relevance to human health.

Utility (Adequacy) for CERHR Evaluation Process: This report has no utility for the evaluation because of the single dose level of genistein or SPI used in the study. The utility of the paper is limited to the mechanistic insight from the assessment of gene expression and the potential for these results to direct future studies.

3.3.5 Non-Rodent Species

Pigs

Chen et al., 2005 *(688)*, supported by the State of Illinois, examined the effect of ingesting a genistein-containing formula on the intestines of piglets. Groups of 8 piglets **[obtained within 48 hours of birth, but exact age at the start of dosing was not specified]** were fed medicated sow-milk replacer formula **[composition of formula not specified]** to which genistein **[purity not specified]** was added at 0, 1, or 14 mg/L. Piglets received the control or genistein-containing formulas at a rate of 360 ml/kg bw/day for 10 days by self-feeding from a tube. **[Based on body weights provided for piglets on the last day of the experiment and reported body weight gain during the course of the study, genistein intake was estimated at ~0, 0.1–0.4, and 2–3 mg/kg bw/day.]** On the 10th day, the piglets received one-third of their daily formula allotment before being killed. Parameters examined in piglets included growth, serum isoflavone levels by LC/MS, intestinal lactase, sucrase, and disaccharose activity, intestinal cell migration, proliferation, apoptosis, electrophysiology, and histomorphology, intestinal expression of *ERα*, *ERβ*, and trefoil factor mRNA, and expression of phospho-src Tyr 416 protein. Data were analyzed by 1-way ANOVA.

Mean ± SD levels of serum genistein were reported at 0.01 ± 0.02, 0.07 ± 0.07, and 2.36 ± 2.26 μM **[2.7 ± 5.4, 19 ± 19, and 637 ± 610 μg aglycone equivalents/L]** in the respective dose group. No genistein treatment effects were detected on body weight gain of piglets or piglet intestinal weight or length. Jejunal villous height, width, and crypt depth did not differ significantly by dose group. There were no detected treatment-related effects on electrophysiological measurements, including ion, glucose, or glutamine transport, in jejunum or ileum. No effects of genistein on jejunal disaccharide,

lactase, and sucrase activities were detected. Reduced enterocyte proliferation was observed in the 14 mg/L genistein group, as noted by PCNA levels that were about half those of the control group. A trend for reduced enterocyte migration was identified in the 14 mg/L genistein group, for which the migration distance was about 20% less than control values. No significant differences were observed for apoptosis in intestinal villi. No significant effects compared to control values were observed for ERα or ERβ expression in jejunum or ileum. There was no detected genistein effect on expression of trefoil factor mRNA in jejunum or ileum, but trefoil faction mRNA was significantly lower (by ~33%) in stomach in both treated groups. No significant effect of genistein treatment on phospho-src Tyr 416 protein expression in jejunum was detected. The study authors concluded that the data on inhibited jejunal enteroctye proliferation and migration provided compelling evidence of genistein bioactivity in the intestine following exposures equivalent to those received by infants fed soy formula.

Strengths/Weaknesses: Strengths of the study included use of 8 piglets/group use of oral exposure, inclusion of two dose levels of genistein, measurement of formula consumption and calculation of genistein intake, the determination of serum genistein levels. In all groups including the controls, and the assessment of multiple endpoints relevant to intestinal development. A weakness of the study is the short exposure duration (10 days), lack of detail regards the content of the formula although somewhat mitigated by measurement of serum genistein levels in all groups, lack of assessment of stability of genistein in the formula, and a lack of evaluation of endpoints relevant to the reproductive system. It is also unclear from the methods as to the number of fields or cells that were evaluated for the semi-quantitative analyses of enterocyte migration and proliferation, villus apoptosis, and morphometric assessments of the villi.

Utility (Adequacy) for CERHR Evaluation Process: This study is of limited utility in determining developmental effects due focus of the study on the small intestine. The study shows that a dose of genistein in the biological range for infants increases caspase-3 while decreasing new cell proliferation (BrdU) in the intestine. Evaluation of the impact of a longer period of exposure to genistein on the intestine would be of interest.

3.4 Experimental Animal Studies of Soy Formula or Other Soy Exposures during Development

3.4.1 Growth, Reproductive System and Endocrine-Related Endpoints

The effects of developmental soy exposure on reproductive endpoints were examined in rats, mice, rabbits, pigs and non-human primates. The majority of studies involve oral exposures. Studies in rats are presented first, in order of female effects followed by male effects and then studies with males and females. Within each section, studies are presented alphabetically by last name of the first author.

3.4.1.1 Rats

Female

Ashby et al., 2000 ***(297)*** examined the effects of post-lactational oral exposure to Infasoy®, an infant soy formula, and animal feeds on uterotropic responses and initiation of puberty in female rats. The uterotropic effects are summarized in **Section 2.2.9.2**. Two separate experiments were conducted. In the first experiment, a control group of 34 rats from 5 litters was fed RM1 **[Rat and Mouse No. 1, a soy-based diet]** and a comparison group of 29 rats from 6 litters was fed AIN-76 (a phytoestrogen-

free diet). In the second experiment, a control group of 49 rats from 9 litters was fed the RM1 diet and a comparison group of 61 rats from 10 litters was given Infasoy® in drinking water. In both experiments, rats were exposed to each diet or treatment on PND 21–55. Body weights and age of vaginal opening and first estrus were monitored. Data were analyzed by ANOVA and ANCOVA, with the litter as the unit of analysis. Results are summarized in **Table 140**. Vaginal opening and first estrus occurred at a younger age in rats fed the AIN-76A compared to the RM1 diet. Body weights of rats fed the AIN-76-A diet were higher on PND 35 but lower on day of vaginal opening. Age of vaginal opening and first estrus were accelerated in the rats given Infasoy® compared to control rats fed the RM1 diets. Body weights of rats given Infasoy® were higher than control rats on PND 46 but were lower than control rats on the day of vaginal opening and first estrus. As explained in greater detail in **Section 2.2.4.2**, uterotropic responses were attenuated by the ERα and ERβ antagonist Faslodex, the aromatase inhibitor anastrozole, and the GnRH antagonist antarelix. The study authors noted that sexual development in rodents can be accelerated by exogenous synthetic or dietary estrogens interacting with tissue ERs or through a centrally mediated increase in endogenous estrogens. The study authors concluded that dietary components other than contaminant synthetic estrogens or phytoestrogens can accelerate sexual development in rodents and may therefore be a factor in precocious human development.

Strengths/Weaknesses: Strengths of the study were use of soy milk, exposures only postnatally (PND

Table 140. The Effects of Diet and Soy Formula Given to Rats on PND 21–50 (Ashby et al., 2000)

Endpoint[a]	*Experiment 1*		*Experiment 2*	
	RM1 Diet (Control)	*AIN-76A Diet*	*RM1 Diet (Control)*	*RM1 Diet + Infasoy®*
Body weight, g				
PND 21	40.8 ± 4.7	40.2 ± 3.7	37.1 ± 5.7	37.3 ± 5.6
PND 35	107.1 ± 9.1	120.5 ± 8.3[b]	106.0 ± 12.0	107.6 ± 17.0
PND 46	N.D.	N.D.	156.2 ± 12.3	165.3 ± 14.4[b]
At vaginal opening	108.6 ± 11.6	97.7 ± 9.0[b]	102.4 ± 13.2	91.5 ± 10.8[b]
At first estrus	N.D.	N.D.	115.2 ± 19.8	108.3 ± 18.7
Age, days				
At vaginal opening	35.1 ± 1.9	31.5 ± 1.2[b]	34.5 ± 2.0	32.4 ± 1.2[b]
At first estrus	38.1 ± 2.8	34.6 ± 3.5[b]	37.1 ± 3.9	35.3 ± 2.7c

[a] Data expressed as mean ± variance **[unspecified but listed as SD in other figures of this report]**.
[b] $P < 0.01$ compared to control.
c $P < 0.05$ compared to control.
N.D. = Not determined.
From Ashby et al., 2000 *(297)*.

21–55), adequate sample size (n=9–10), and use of the litter as the experimental unit. Weaknesses include the lack of information on phytoestrogen content of the chow, the failure to monitor liquid consumption, resulting in unknown exposure to soy, the use of a soy-based chow (RM1) with experimental exposure to Infasoy, resulting in 2 sources of soy exposure, the use of only 1 soy formula

dose level, and the lack of clarity on how animals were chosen for analysis (presumably all females from all litters evaluated).

Utility (Adequacy) for CERHR Evaluation Process: This study is of no utility for the current evaluation. Even though rats were treated with soy infant formula, the treatment began after lactation on PND 21.

Chang et al., 2007 ***(689)*** **[source of funding not stated]**, investigated the effects of soy isoflavone diet on uterotrophic responses in rats initially exposed as weanlings or adults. Female Sprague-Dawley rats (purchased from Daehan Biolink, Eumsung, Republic of Korea) were randomly assigned to one of eight groups of nine rats each based on body weight: four groups of juveniles, and 4 groups of adults. Test diets were prepared using AIN purified diet with isoflavones added at 0% (Control), 0.02% (LI), 0.1% (MI), and 0.2% (HI). Aglycone-type isoflavone powder was manufactured from soybean germ containing 32.18% isoflavones (3.76% genistein, 19.33% daidzein, 9.09% glycitein). All rats were fed test diets for six weeks beginning when they were either weanlings **[age not stated]** or when they were 16 weeks old. After 5 weeks, the rats were housed in metabolism cages for urine collection. Urine was measured (total volume) and analyzed for excreted genistein and daidzein using HPLC. After six weeks the rats were killed and the uteri were fixed in NBF, histopathology slides were stained with H&E, and photomicrographs were taken of the endometrium. A double-blind examination was performed for the presence of hyperplasia. Hyperplasia was scored on a 3-point scale: 0, no specific change; +, obviously increased cellularity; ++, obviously increased cellularity and formation of a double layer of endometrial epithelial lining cells. Analysis of variance was performed on mean values; when significant, Duncan's multiple range test was applied. Differences between the juvenile and the adult groups were compared by Student's *t* test. $P < 0.05$ was considered significant.

In juvenile rats, feed intake of the MI and the HI groups was significantly lower than the Control and the LI groups; in adult rats there were no significant differences in feed intake between the groups. The final body weights and the amount of weight gain of the juvenile MI and HI groups were significantly lower than the juvenile Control and LI groups. The adult HI group gained significantly less weight than the Control group. As the levels of dietary isoflavones rose, the urinary excretion of daidzein and genistein was significantly higher in the juvenile MI and HI groups compared to the juvenile LI group. For the adult rats, the amounts of urinary excretion of daidzein and genistein were significantly lower with higher levels of dietary isoflavones. The urinary recovery rates for daidzein and genistein were significantly lower in the MI and HI groups compared to the LI group for both the juvenile and the adult rats. The recoveries of daidzein and genistein in the juvenile rats were significantly lower than in the adult females.

Calculated urinary recoveries of genistein for the LI, MI and HI groups were 8.8%, 3.1% and 2.2% respectively, in the juvenile rats and 22.0%, 8.8% and 7.9%, respectively, in the adult rats. The results for daidzein showed similar tendencies **[data not presented]**. Elimination rates were also lower and the excretion half-life values were longer in juvenile rats than in adults. Dietary isoflavones did not cause histological changes in the epithelial cells of the juvenile rats. The uterine sections from the adult rats showed epithelial lining cells with typical enlarged nuclei and increased cellularity. The incidences of hyperplasia in the LI, MI, and HI groups of adult rats were 44%, 25% and 38% of cases, respectively - there was no linear response.

Authors' conclusion: Isoflavones were more bioavailable in adult rats than in juvenile rats. Therefore, soy isoflavones supplementation may not act as an endocrine disrupter during the growth period, but may induce a phytoestrogenic effect on the uterus of adult rats.

Strengths/Weaknesses: Strengths of the study are that the composition of the dosing material was well-characterized. Weaknesses are that the evaluation of uterine hyperplasia was not well-described and that the assessment of other endpoints related to reproductive function or tissues was weak.

Utility (Adequacy) for CERHR Evaluation Process: The study has no utility for the evaluation.

Chen et al., 2008 *(690)*, supported by ARC CRIS, compared the effects of dietary exposure to soy protein isolate, whey protein hydrolysate, and rice protein isolate on tibial bone mineral density and bone mineral content in intact or ovariectomized rapidly growing female rats relative to animals fed casein; two experiments were conducted. *Experiment #1*: Pregnant Sprague-Dawley rats (Harlan Industries, Indianapolis, IN) were fed AIN-93G diets with casein as the sole protein source beginning gestation day 4; their offspring were weaned on PND 21 and fed the same diet until PND 55 **[bedding not described]**. On PND 55, half of the female offspring were ovariectomized (OVX) and all females (OVX and intact) were subdivided into 8 groups and fed one of four pelleted diets made with: casein (CAS, control), soy protein isolate (SPI), whey protein hydrolysate (WPH), or rice protein isolate (RPI) (n=6/group). All diets were made according to the AIN-93G diet formula except that corn oil replaced soy oil and the protein source was CAS (Fonterra), WPH 917 (Fonterra), RPI (proprietary method), or SPI (Clinical blend 670); the percentage of protein content of each diet was 20%. After 14 days of feeding, the rats were killed and blood, femur and tibia were collected; bone mineral density (BMD) and bone mineral content (BMC) were measured. *Experiment #2*: Twenty-four Sprague-Dawley rats were fed AIN-93G diet made with either casein or soy protein isolate from PND 30 through PND 69. On PND 55, rats were ovariectomized and the two diet groups were further divided into groups receiving polyethylene glycol vehicle or 17β-estradiol (E2) (n=6/group). A mini osmotic pump implanted subcutaneously administered 17β-estradiol; the final dose of E2 was 5 μg/kg/day. All animals were killed on PND 69 and blood and tibia were collected. pQCT scans were performed on formalin-fixed tibia from each rat; bone mineral density (BMD) and bone mineral content (BMC) were measured. The serum bone formation marker alkaline phosphatase (ALP) was measured by a colorimetric assay; the serum bone formulation marker osteocalcin and the serum bone resorption maker RatLaps were measured by ELISA; tibial bone total RNA was extracted. Bone marrow cells were harvested from casein-diet control rats and alkaline phosphatase staining was performed; cell RNA was isolated and real-time RT-PCR was performed. One-way analysis of variance (ANOVA) was utilized to analyze diet effects and two-way ANOVA for analysis of SPI/CAS+E2 interactions followed by Student-Newman-Keuls post hoc analysis for multiple pair-wise comparisons between treatment groups.

Experiment #1: For intact (not OVX) rats: No diet effects were seen on either food intake or body weight gain. Total trabecular and cortical BMD and total BMC of the tibia were all greater for rats in the SPI group compared to the control group (casein-fed); total tibial BMD was greater in the WPH group. There was no difference in pQCT parameters when the PRI group was compared with the CAS group. When all for groups were compared, the SPI group had a greater BMD. For the OVX rats: The trabecular BMD and total BMC were significantly lower for the OVX control group compared to the intact control. Total tibial BMD and total tibial BMC for the OVX SPI group were both higher than the

OVX control group; total tibial BMD, trabecular BMD and total tibial BMC were significantly higher in the OVX WPH group compared to the OVX control. There were no significant differences in tibial BMD when OVX RPI was compared to the OVX control, but the BMC was significantly higher. OVX SPI had significantly higher trabecular BMD and total BMC compared to either OVX WHP or OVX RPI, and the values for the OVX SPI group were comparable to the intact SPI group. There were no significant differences in tibial cortical BMD among all four OVX rat groups. *Experiment #2*: Plasma E2 values were higher in the OVX+E2 group compared to the OVX control. In the OVX SPI group, total tibial BMD, trabecular BMD and total BMC were all significantly higher than the OVX CAS (control) group. All pQCT measures were higher in the OVX CAS+E2 group when compared to the OVX control. E2 replacement to physiological levels had greater effects on tibial BMD and BMC compared to SPI feeding in OVX rats; with the combination of SPI and E2 replacement, SPI-feeding lessened the E2 effect on tibia trabecular BMD and tibial total BMC. A similar pattern of effects of CAS, SPI, CAS+E2 and SPI+E2 was seen on total tibial BMD; there was no statistical difference between OVX CAS+E2 and OVX SPI+E2. Replacement of E2 also resulted in a higher cortical BMD; there was no effect of SPI on cortical BMD when compared to the OVX CAS control group. Osteocalcin and ALP were higher and RatLaps was lower in OVX animals fed the SPI diet compared to animals fed the CAS (control) diet. Serum bone markers and serum bone resorption markers were both lower in the OVX CAS+E2 group compared to the OVX control. Serum osteocalcin in the OVX SPI+E2 group was significantly higher than the OVX CAS+E2 group, but were significantly lower than the OVX SPI group. Serum ALP values in the OVX SPI+E2 group were intermediate between the OVX SPI group and the OVX CAS+E2 group. The bone resorption marker RatLaps in the OVX SPI+E2 group was significantly higher than the OVX CAS+E2 group. In total RNA extracted from tibial bone, ALP mRNA was significantly higher in the OVX SPI group compared to the OVX control and osteocalcin mRNA tended to be higher in the SPI group compared to the CAS (control) group, but was not statistically significant. In the OVX CAS+2 group, both ALP mRNA and osteocalcin mRNA in bone were greater compared to the OVX CAS group. RANKL mRNA expression in tibial bone was lower in both the OVX SPI diet group and the OVX SPI+E2 group compared to the OVX control. There was no effect of E2 itself on RANKL, and RANKL mRNA expression in the OVX SPI+E2 groups. Whereas CAS+E2 marginally suppressed ERα and had no statistical effects on ERβ gene expression, expression of both receptors was dramatically down-regulated by feed SPI diet. When SPI diet and E2 treatment was combined in OVX rats, ERα and ERβ gene expression levels were in between the OVX SPI and the OVX CAS+E2 group, but remained lower than the OVX CAS group. Compared to serum from CAS (control) intact rats, 2.5% serum from intact SPI group rats dramatically promoted osteoblast differentiation by alkaline phosphatase staining. Increased gene expression of alkaline phosphatase and osteocalcin from cells treated with 2.5% serum from intact SPI group rats was observed. 2.5% serum from soy protein isolate-fed rats had greater effects on osteoblast differentiation than the positive control (osteogenic medium).

Authors' conclusion: The data suggest beneficial bone effects of a soy diet in rapidly growing animals and the potential for early soy consumption to increase peak bone mass.

Strengths/Weaknesses: Strengths of the study include the use of dietary exposure, use of a soy-free diet, the diets were designed to be isonitrogenous and isocalorific with a protein content of 20%, the period of exposure for experiment 1 was appropriate for assessment of effects on development, and evaluation of multiple endpoints relevant to the bone. Weaknesses of the study include the lack of detailed analysis of the diet contents, food intake was not reported, circulating levels of isoflavones were not determined,

and there was no dose-response to different protein levels in the diet. It is difficult to know exactly what the animals were being exposed to in the study. The study was limited to females only.

Utility (Adequacy) for CERHR Evaluation Process: This study has no utility since the study does not sufficiently define the dose levels administered/exposures obtained for the purposes of the present evaluation.

Hong et al., 2006 *(691)*, supported by the Korean Research Foundation, the Research Project on the Production of Bio-organs, the Ministry of Agriculture and Forestry, the Ministry of Education and Human Resources Development (MOE), the Ministry of Commerce, Industry and Energy (MOCIE), the Ministry of Labor (MOLAB), and the Canadian Institutes of Health Research, investigated the altered gene expression by estrogen and endocrine disruptors (EDs) using microarray technology in the uterus of immature rats. Female Sprague-Dawley rats, two weeks of age (Orient Co, Ltd, Gyeonggi-do, Korea) **[feed and bedding not described]** were injected sc with: diethylstilbestrol (DES, 500 μg/kg BW), octyl-phenol (OP, 600 μg/kg BW), nonyl-phenol (NP, 600 μg/kg BW), bisphenol-A (BPA, 600 μg/kg BW), or genistein (40 mg/kg BW) as a single daily dose for 3 days (n=5 rats/group). All chemicals were dissolved in corn oil; estradiol (E2, 40 μg/kg BW) served as the positive control (n=3) and corn oil as the negative control (n=3) **[purity of the chemicals not stated]**. All rats were killed 24 hours after the last injection and the uterus was collected and used for microarray and RT-PCR analysis. To confirm altered gene expression, 40 adult female rats **[age not stated]** were randomly assigned to 4 groups based on their estrous cycle stage and killed immediately; the uterus was collected and used for RNA extraction. The data were analyzed by non-parametric procedure of the Kruskal Wallis test, followed by Dunnett's test for two-pair comparisons.

The expression levels of 555 genes (7.42% of 7636 genes spotted) were enhanced by more than 2-fold following treatment with E2 (positive control). Elevated expression levels of the genes were observed for DES (9.01%), OP (8.81%), NP (9.51%), BPA (8.26%) and genistein (9.97%) in the immature rat uterus. The expression levels of representative genes were confirmed in the adult tissues by real-time PCR. The mRNA levels of these genes detected by real-time PCR were greater during the follicular phase when E2 level is elevated during the estrous cycle of adult female rats.

Authors' conclusion: The results indicate a distinct altered expression of responsive genes following exposure to estradiol and estrogenic compounds, and implicate distinct effects of endogenous estradiol and environmental endocrine disrupting chemicals in the uterus of immature rats.

Strengths/Weaknesses: Strengths relate to the use of several putative estrogenic chemicals to evaluate effects on rat uterine gene expression using microarray analysis. Subsets (500-700) of the ~7600 genes probed were affected by estradiol, DES, octyl-phenol, nonyl-phenol, BPA and genistein. Weaknesses were the rats were given sc routes of exposure, and single doses of each estrogenic chemical. Rats in different stages of the estrous cycle were used as positive controls, however, effects were difficult to compare with those observed with estrogenic chemicals, and purity of genistein is unknown. Also, age of rats was unclear, therefore an age-exposure effect cannot be assessed. Design and interpretation of microarray analysis may not have been optimal. Genes measured that were affected by estradiol and DES were not the same genes affected by genistein, providing a striking mechanistic disconnect between uterine actions of estradiol and genistein.

Utility (Adequacy) for CERHR Evaluation Process: This study is of limited utility for the evaluation process because this provides mechanistic evidence that genistein may not have putative estrogenic effects by acting in the same manner as estradiol. However, because single doses of the agents were given, it cannot be ruled out that some chemicals were tested out of their optimal concentration as regards estrogenic effects.

Liu et al., 2008 ***(692)***, supported by the Chinese Nature & Science Grant and Chinese Eleventh Five-Year National Science & Technology Supporting Plan for Food Safety Key Technology, examined the effects of lactational exposure to soy isoflavones administered by gavage on the rat female reproductive system. Female Sprague-Dawley rats (obtained from Vital River Laboratory Animal Institute, Beijing, China; 9-weeks old at mating) and their litters were randomly assigned to six treatment groups on postnatal day (PND) 4. Each group contained 7 females and their litters. Animals were fed a Soy-and alfalfa-free diet (SAFD) **[bedding was not described].** The dams were treated daily via gavage with 0 (control), 10, 50, 100, 150 or 200 mg/kg body weight/day of soy isoflavones (80%, genistein:daidzin:glycetin=13:5:2) **[ratio assumed to be reflective of composition in soy formula]** from PND 5 to PND 10 **[basis of dose selection not described]**. Distilled water served as the vehicle. On PND 11, 10 female pups in each group were randomly selected and killed; each litter was represented by at least one pup. The uteri and ovaries were weighed; one ovary and one uterine horn were fixed in NBF, embedded in paraffin, sectioned and stained with H&E. An examination for morphologic changes, including uterine gland numbers and endometrial thickness was performed.

Immunohistochemistry was carried out using DAB **[assumed to be diamino-benzadine]** as the chromogen. Serum estradiol and progesterone concentrations were determined for 10 litters in each group **[only seven litters assigned to each group, this may be 10 pups]** using an ELISA assay. RNA of the ovary and uterus was isolated using Trizol reagent. The RNA was checked for degradation by agarose gel electrophoresis; the quality of total RNA was determined spectrophotometrically. Polymerase chain reaction (PCR) analysis was conducted. Bands were photographed and scanning densitometry was used to quantitate the intensity; the densitometric value for each biomarker was normalized to β-actin expression. Analysis of variance was used to analyze body weight, organ weight, endometrial thickness, estradiol and progesterone concentrations, and densitometric values for RNA expression followed by testing for variance homogeneity. Dunnett's test (two-sided) was used to compare differences between the isoflavone treatment groups and the control group. A two-tailed P-value of 5% ($P<0.05$) was considered statistically significant.

There were no statistically significant differences in pup body weights on PND 4 and PND 11. The absolute and relative weights of the uterus here were a statistically significantly higher for the 150 and 200 mg/kg body weight treatment groups and the ovary for the 100 mg/kg body weight and higher treatment groups compared to the control group. The litters in the control group showed a single layer of cuboidal or low columnar cells lining the uterine lumen; however, the uterine lumen from litters in the100 mg/kg body weight or above groups was visibly thicker. Litters from the 150 and 200 mg/kg body weight treatment groups had a statistically significantly greater endometrial thickness than animals from the control group. There was no effect of treatment on the number of uterine glands. Soy isoflavone-treated groups had greater proliferating cell nuclear antigen staining of ovary and uterus in comparison to the control group. The intensity values for proliferating cell nuclear antigen staining of ovary and uterus in the 150 and 200 mg/kg body weight groups were statistically significantly

higher than the control group. Estradiol levels in the 50, 150, and 200 mg/kg body weight groups were statistically higher than the controls. Serum progesterone concentrations were lower in the soy isoflavone-treated groups, especially in the 100 and 200 mg/kg body weight groups. Ovaries in the 150 and 200 mg/kg body weight treatment groups had statistically significantly lower densitometric values for progesterone receptor mRNA expression when compared to the control group. The 150 and 200 mg/kg body weight treatment groups had statistically significantly higher densitometric values for estrogen receptor mRNA expression for the ovary, and statistically significant lower values for the uterus.

Authors' conclusion: Lactational exposure to isoflavones could result in estrogen-like actions on the reproductive system of neonatal female rats. The mechanism may be, at least, involved with modifications of hormone production and steroid receptor transcription in the reproductive system.

Strengths/Weaknesses: Overall, the experimental design of this study was good. Specific strengths include adequate sample size and control for litter effects, use of an oral route of administration that is relevant to human consumption, and testing of a relatively wide range of dose levels. Weaknesses are that it is unclear whether the mRNA for ER and PR is for ERα or ERβ and PR-A or PR-B, respectively. In addition, it is difficult to interpret the significance of the results because the estrogen/progesterone status of the mothers/breast milk was not known.

Utility (Adequacy) for CERHR Evaluation Process: This study is of high utility for the evaluation process.

Peterson et al., 2008 ***(693)***, supported by the University of Missouri Research Board, examined the skeletal and reproductive system effects in female rats introduced to a diet containing soy proteins as weanlings. The study was conducted in two experiments, 8-9 animals per group were used for each experiment. Female Harlan Sprague-Dawley weanling rats (Indianapolis, IN, ~ 3 weeks old) were randomly assigned to one of four dietary regimens. All diets were nutritionally complete, following a modified AIN-93G formulation in which the protein (200 g/kg diet) was provided as either casein (casein-based control group) or soy protein isolates containing either 0.11 (Low IF group), 2.16 (Med IF group), or 3.95 (High IF group) mg total aglycone isoflavones/g protein. Aglycone genistein contributed 0.5, 1.27 and 2.23 mg/g diet to the Low IF, Med IF, and High IF diets, respectively. Animals remained on the test diets for 8 weeks (the time frame corresponds to childhood and adolescence in the female human life span). Rats were weighed weekly, beginning at week 5, pair-feeding was employed to correct for the lower intakes of the High IF group. Vaginal smears were taken daily. At the end of 8 weeks, when the animals were in diestrus, they were killed and blood was collected for determination of serum genistein (by HPLC) and E_2 (using single radioimmunoassay - RIA) as well as estrogenic activity (by bioassay using estrogen-responsive MCF-7 human breast cancer cells). The uteri and ovaries were weighed; the tibias were analyzed for mineral content and bone histology/histomorphology; the remaining carcass was used to estimate skeletal density using dual-energy x-ray absorptiometry (DXA). The data from both experiments were pooled with the exception of estrus cycle and reproductive tissue measurements and E_2 and estrogenic activity determinations which were performed in only one experiment. Data were analyzed by one-way analysis of variance (ANOVA) and/or paired t tests. Group means were consider significantly different at $P<0.05$ as determined by the protected least-significant difference technique. Repeated measures ANOVA was applied to the growth and feed intake data. For the estrous cycle phase data, χ^2 analysis was performed to assess the effect of diet on phase length. For E_2 and estrogenic activity measurements, all data were log transformed before ANOVA and paired t tests.

Daily feed intake of the High IF group diverged from the other diet groups during weeks 3 and 4, pair-feeding was employed to equalize intakes until the end of the 8-week experimental period. Despite the equalization, the Low-IF group grew at a significantly slower rate than the other diet groups. Animals in the Med IF and High IF groups had significantly higher concentrations of serum genistein than animals in the casein-based control or Low-IF groups. Total serum E_2 concentrations were consistent with the diestrus phase of the estrous cycle. Overall, the serum concentration of E_2 was significantly different among groups; when only the soy-fed groups were compared, this difference became even more significant - the mean E_2 serum concentrations of the High-IF group was significantly lower than the Low-IF group, however there was no change in circulating estrogenic activities. Rats fed the Low-IF diet exhibited a delayed vaginal opening compared to the other experimental diets. All rats cycled throughout the entire experiment. There were no differences seen for length of estrous cycle or number of days spent in each phase. The uteri of the casein-based control group weighed significantly less than uteri of the rats in the MED-IF and High-IF groups; ovarian weights did not differ between groups. There were no differences between groups for bone mineral content or bone mineral density. Fat-free dry and ashed tibial weights of the Low-IF group were significantly less than the other diet groups, however when corrected for body weight and fat-free bone weight, these differences disappeared. Total tibial calcium content was significantly less in the Low-IF group, while total tibial phosphorus content was not significantly different among diet groups. Qualitative assessment of the tibial morphology and bone histomorphology revealed no remarkable differences between diet groups.

Authors' conclusion: Soy consumption during peripuberty has no deleterious effect on either longitudinal bone growth or bone mineralization. The serum estrogenicity and E_2 data, considered together, suggest that in young animals there are homeostatic mechanisms in place to defend certain target tissues from exposure to the superphysiological concentrations of estrogenic compounds achieved by the ingestion of exogenous (plant-derived) estrogens. Since the uteri of the animals fed the soy protein diets responded partially to the isoflavone levels, the same degree of protection may not be conferred to all target tissues, presumable because of differences in ER profiles.

Strengths/Weaknesses: Strengths of the study include exposure to isoflavones in the diet, the assessment of a dose-response, use of an isoflavone-free diet, description of the contents on the diets and amounts of aglycone (as determined by the manufacturer), measurement of serum genistein, estradiol, and estrogenic activity, and multiple endpoints of bone health including histomorphometry were included. A weakness of the study is the exposure period which was selected to cover childhood and adolescence so dosing started at weaning and continued for 8 weeks, however, it did not include lactation interval which is important for the assessment of effects on development. The study did not include male rats.

Utility (Adequacy) for CERHR Evaluation Process: This study alone has no utility for the evaluation since the exposure period did not cover all the intervals that would be appropriate for the assessment of effects on development. The study may have more utility when considered in the context of other studies assessing bone health.

Males

Akingbemi et al., 2007 ***(694)***, conducted this study at Auburn University's College of Veterinary Medicine with the research supported by an Auburn University fund for faculty development. These

studies examined the effects in male rat offspring following maternal ingestion of a isoflavone-containing diet during gestation and lactation .The first study was a diet-administered study using time bred Long-Evans dams (9 per group). The concentration of isoflavones in the experimental diets were 0, 5, 50, 500, or 1000 ppm based on the assayed content of genistein and daidzein and calculation of the equivalent aglycone as specified by the manufacturer. Presumed pregnant dams were fed diets from GD 12 until weaning at day 21 postpartum (PND 21 for the offspring.) The date of birth was considered PND 1. **[Age of the animals at study initiation was not provided.]** The adult female body weight at study initiation was approximately 250 grams.

Pregnancy outcome was assessed on PND 1: litter size, pup weight, and pup sex ratio. Male pup body weights and anogenital distance were measured on PND 5. To minimize within-group variations in treatment effect due to individual metabolic profiles of dams, male pups were pooled together on PND 5 and reassigned to dams in the same group until weaning. Subsequently male pups were randomly selected for analysis on PND 21(8-11 male pups per group). Analysis included measurement of genistein and daidzein levels in the serum, liver, testis and assessment of testicular steroidogenesis. All the remaining male offspring were placed on soy-free diets from PND 21 to PND 90 and allowed to attain sexual maturity at PND 90, when they were sacrificed and analyzed for serum, hormone concentrations (testosterone, T; luteinizing hormone, LH; and endogenous estrogen, E_2), epididymal and accessory sex organ weights, testicular and Leydig cell T production, and ERα gene expression (4 rats/group in 0, 5 and 1000 ppm groups) in the prostate gland.

Data were presented as mean ± SD. Data were analyzed by one-way ANOVA followed by Dunnett's test for multiple group comparisons. Differences of $P<0.05$ were considered significant.

Feeding soy-based diets to pregnant dams did not affect litter size or the pup weights and sex ratios of offspring. Male pup body weights from dams fed the 5 and 50 ppm diets were significantly heavier on PND 5 than the control pups. The anogenital distance (ANG) was also significantly greater in PND 5 male offspring from the 5, 50, and 500 ppm groups. These differences were not apparent when normalized for the pup body weights. All male rats' body weights except the 500 ppm diet group were heavier than control at PND 21, but these differences were no longer apparent at PND 90. Absolute paired testis weight was significantly heavier in PND 21 pups from all diet groups vs control; this effect was significant only in the 50, 500 and 1000 ppm groups after adjusting for body weight (relative testis weight). At PND 90, there were no differences in male body weights from any group, but absolute testis weight was significantly higher in the 50, 500, and 1000 ppm diet groups and was significantly lower in the 50 ppm group when corrected for body weight (relative testis weight). Exposure of male rats did not affect weights of the epididymis, dorsolateral and ventral prostate, bulbourethral glands and seminal vesicles in adult rats at PND 90.

There were large variations in the serum levels of genistein and daidzein (unconjugated or glucuronide metabolite) within groups in the PND 21 male rats. The concentrations of genistein and daidzein glucuronides were severalfold higher (about 25 times for genistein and 18 for daidzein) than the unconjugated aglycone compounds. The liver and testis concentrations were less variable. The concentrations of unconjugated genistein and daidzein in the liver were about 10-fold higher than their levels in the testis, and higher daidzein levels than genistein were detected in both tissues. For both genistein and daidzein, the 50, 500 and 1000 ppm groups showed significantly higher concentrations

in the liver and testis vs the controls. These observations imply that phytoestrogens in the maternal diet have the capacity to cross maternal tissue barriers to reach the fetus and neonate.

Serum testosterone (T) levels were significantly higher in the 5 ppm group vs control in PND 21 rat pups, because of significantly higher Leydig cell T production. In the 500 and 1000 ppm diet groups, serum T levels were significantly lower whereas serum androsterone, AO, was significantly higher in the PND 21 rat pups. The rates of AO secretion by Leydig cells were significantly lower in the 50, 500 and 1000 ppm diet groups vs control. Serum T and AO levels were unchanged vs control in the 50 ppm group.

Serum LH concentrations were similar in male rats from all diet groups in PND 21 male offspring. Serum LH levels were significantly higher only in the 50 ppm group vs control in PND 90 adult male rats. Serum E2 levels were similar in all groups in PND 90 male rats suggesting according to the authors that the exposures achieved in this study did not affect Leydig cell aromatase activity.

Adult male rats (PND 90) from dams fed 5 ppm diet showed significantly lower testicular T production vs control although this was not reflected in the serum T levels. The authors suggested that it is possible that suppression of androgen biosynthesis was not profound enough to lower serum T levels or that changes in serum T levels were masked by the pulsatile and circadian rhythm of androgen secretion *in vivo*. PND 90 males from the 1000 ppm group had significantly higher serum T levels and significantly higher testicular T secretion. T production per Leydig cell was significantly lower at the 500 and 1000 ppm groups. The 50 ppm diet did not affect serum T concentrations and Leydig cell T production.

At 5 and 1000 ppm groups, there was a significant lowering of ERα mRNA levels of the ventral prostate of PND 90 rats. Modulation of gene expression was confirmed by immunoblot analysis, demonstrating significantly lowering vs control of ERα protein.

Four *in vitro* analyses were also conducted to assess genistein action on immature Leydig cells from PND 35 Long Evans rats.

In the first analysis, control untreated and genistein-treated Leydig cells were incubated in media containing a maximally stimulating dose of ovine LH (100 ng/ml) in the 3-h posttreatment period. In the second analysis, Control untreated and genistein-treated Leydig cells were incubated in media containing steroid intermediates as T precursors in the 6-h posttreatment period. In the third analysis, Leydig cells were incubated with the antiestrogen ICI 182,780 for 3 h before genistein treatment. For experiments I–III, Leydig cells were treated with genistein for a period of 24 h, and T production was assayed in aliquots of spent media by RIA. To investigate changes in gene expression, Leydig cells were harvested after genistein treatment and processed to obtain total RNA for analysis by RT-PCR and whole cell lysate for immunoblotting. In the fourth analysis, Leydig cells were cultured with and without genistein for 18 h followed by incubation in media containing [3H]thymidine in the 3-h posttreatment period. Testosterone secretion by immature Leydig cells lowered on exposure to 0.1nM genistein *in vitro* (control: 175 ± 5 vs. 117 ± 3 ng/10^6 cells per 24 h, indicative of direct phytoestrogen action.

Overall the authors' state that their data indicate that exposure to phytoestrogens in the perinatal period modulates androgen biosynthesis in the adult rats' testis. The levels of genistein and daidzein

in the blood of the male offspring in this study are comparable to levels that were measured in fetuses of mothers eating Western diets and infants fed soy-based formulas. Feeding a low phytoestrogen diet (5 ppm) stimulated Leydig cell T production in prepubertal male rats. According to the authors, increased Leydig cell T production at this 5 ppm dose level, may explain previous observations that exposures to low genistein doses stimulate spermatogenesis in prepubertal male rats as well as alleviate disturbances of spermatogenesis in mice deficient in E2 production. The 500 and 1000 ppm diets caused a lowering in serum T and higher AO levels.

These phytoestrogen effects have implications for male reproductive function. For example, androgen insufficiency during the period of reproductive tract is thought to be associated with congenital malformations of the male urogenital tract. Phytoestrogen-induced increases in serum T levels have potential implications for pubertal development. According to the authors, there is evidence that prepubertal males are taller at younger ages more than ever before, suggesting earlier maturation. Elevated serum T levels have also been linked to increased risk of testicular germ cell tumors in human subjects. Data from the present study indicates that the perinatal period is a sensitive window for phytoestrogen regulation of Leydig cell differentiation and testicular steroidogenesis.

Strengths/Weaknesses: Strengths of the study include use of dietary exposure, multiple dose levels, characterization of diet, appropriate period of exposure, and measurement of genistein and daidzein in the offspring. The testosterone levels appear to have uncharacteristically low variability, and supporting data from the measurement of testicular testosterone levels would have been useful to confirm the systemic testosterone changes. The lack of concordance of *in vivo* and *in vitro* in relation to potential effects of genistein on testosterone production is a weakness.

Utility (Adequacy) for CERHR Evaluation Process: The study has limited utility for the evaluation process.

Atanassova et al., 2000 ***(621)***, supported by the European Center for the Ecotoxicology of Chemicals and AstraZeneca, compared the effects in rats of maternal consumption of a soy-free or soy-containing diet during pregnancy and lactation on pubertal spermatogenesis and long-term changes in the reproductive system of male offspring. As part of this study, adult female Wistar rats were fed standard diets (15.5% soy meal) or soy-free diets (soy substituted by fishmeal and cereal content increased from 64 to 78%) for 3 weeks prior to mating and through mating, pregnancy, and lactation. Male offspring of rats fed soy-free diets were maintained on soy-free diets from weaning until termination. An unspecified number of males born to mothers on the soy-free diets received sc injections of 4 mg/kg bw/day genistein **[purity not specified]** in phosphate-buffered saline vehicle on PND 2–18, and that part of the study is discussed in **Section 3.3.** Males from the soy-free control group were compared to males in the standard diet control group. **[The total number of rats treated was not stated, but 7–14 rats/group were evaluated.]** On PND 18 and 25, rats were killed and testes were fixed in Bouin fluid. Testicular cell numbers and seminiferous tubule lumen formation were determined by standard point counting of cell nuclei. Apoptosis was assessed by DNA fragmentation detected by *in situ* DNA 3′-end labeling. Plasma FSH and inhibin B were measured by RIA and enzyme-linked immunosorbent assay (ELISA), respectively. In addition, mating and fertility were examined in adult rats (80–90 days old) by placing them in a cage with an unexposed female for 7 days. Statistical significance was determined by ANOVA.

Table 141. Effects of Neonatal Exposures to Soy-Free Diet or Genistein on the Reproductive System of Male Rats (Atanassova et al., 2000)

Effect	*Soy-Free Control compared to Standard Diet Control*	*Genistein, 4 mg/kg bw/day (Soy-Free Diet) compared to Soy-Free Control*
Germ cell apoptotic index, PND 18	↔	↑
Germ cell apoptotic index, PND 25	↔	↔
Seminiferous tubule lumen formation, PND 18	↔	↓
Plasma inhibin B, PND 18	↔	↔
Sertoli cell nuclear volume/testis, PND 18	↑	↔
Plasma FSH, PND 18	↔	↓
Plasma FSH, PND 25	↔	↔
Spermatocyte/Sertoli cell nuclear volume, PND 18	↑	↓
Spermatocyte/Sertoli cell nuclear volume, PND 25	↑	↔

↑,↓,↔ Statiscally significant increase, decrease, or no significant effect.
From Atanassova et al., 2000 *(621)*.

Results and statistical significance for endpoints characterizing pubertal spermatogenesis in 18- and 25-day-old rats are listed in **Table 141**. The study authors noted that the increase in spermatocyte nuclear volume per Sertoli cell nuclear volume in rats fed soy-free compared to standard diets on PND 18 suggested that dietary soy retarded an aspect of pubertal spermatogenesis. For parameters also assessed on PND 25, the only significant effect that remained was the increase in spermatocyte nuclear volume per Sertoli cell nuclear volume in soy-free compared to standard-diet controls. Testis weights in adult rats (90–100 days old) from the soy-free group were significantly (8%) higher compared to rats in the standard-diet group, and testis weights of rats in the genistein group were similar to those in the soy-free group. Animals in the soy-free control group were not mated.

In a larger study reported in this paper, body weight, testis weight, and plasma FSH levels were compared in 24 litters from soy-free groups and 29 litters from standard-diet groups. Male rats were evaluated at 90–95 days of age. Rats in the soy-free group had significantly higher body (5.7%) and testis weights (3.6%) and significantly reduced plasma FSH levels (11.1%).

The study authors concluded that "the presence or absence of soy or genistein in the diet has significant short-term (pubertal spermatogenesis) and long-term (body weight, testis size, FSH levels, and possibly mating) effects on males."

Strengths/Weaknesses: A strength of this study was the large numbers of litters examined (n=24–29). Weaknesses included lack of comparison of dietary nutrients, examination of only males, and lack of determination of reproductive capability. Exposure through chow is a strength in its relevance to human exposure and a weakness in permitting only imprecise estimates of dose levels.

Utility (Adequacy) for the CERHR Evaluative Process: The endpoints examined in this study are of limited utility alone in determining developmental effects.

Górski et al., 2006 ***(695)***, **[support not indicated]**, evaluated the reproductive tissue and hormone effects in male offspring of rats that were fed a diet containing soy during pregnancy and lactation. A second experiment evaluated the response of adult males to a maintenance soy diet over shorter and longer periods of time. Wistar rats **[source not stated]** were fed experimental diets that contained solvent soybean meal (SBM) and soy protein concentrate (breeding diet, experiment 1) or SBM only (maintenance diet, experiment 2) as the main sources of supplementary protein. In soy-free control diets, whey protein concentrate and whole egg powder were substituted for soy products **[purity not stated for any compound]**. The total concentrations of isoflavones were estimated to be approximately 311 μg/g and 264 μg /g in the experimental breeding and maintenance diets, respectively. *Experiment 1*: Two groups of females (F_0 generation) were fed a soy meal-containing (E1) or a soy-free (C1) breeding diet during the growing period, gestation and lactation **[bedding not described]**. Twenty-four males (F_1 generation) from each group were selected for the experiment. Twelve males from each group were killed at weaning on postnatal day 22 (PND 22) and the other 12 per group were fed the respective maternal diet until killed on PND 60. Terminal body weight, as well as testis, epididymis, and prostate weights were recorded. Blood was collected and plasma levels of luteinizing hormone (LH), testosterone and prolactin were determined by radioimmunoassay (RAI). *Experiment 2*: Sixty-day old rats (fed standard chow from weaning until placed in the experiment) were divided into two groups: fed soy meal-containing (E2) or soy free (C2) maintenance diets (n=20/group). Ten rats per group were killed on PND 160 and the remainder was killed on PND 280; organ weights were recorded and plasma hormone levels were determined as in Experiment 1. The significance of differences in the organ weights and the plasma hormone concentrations were determined by the non-parametric ANOVA rank Kruskal-Wallis test within each age group.

Experiment 1: No effect of diet was seen for the PND 22 rats for any parameter evaluated. Diet did not significantly affect reproductive organ weights on PND 60. On PND 60, LH and prolactin plasma concentrations were significantly higher in the E1 males compared to C1 males; plasma testosterone levels were slightly higher in the E1 males compared to controls on PND 60, but this difference was not statistically significant. *Experiment 2*: On PND 160, rats fed the E2 diet had significantly lower mean terminal body weights and absolute organ weights compared to the control rats. Mean relative testis and epididymal weights were higher for the E2 rats than those fed the C2 diet; prostate weight was not affected by diet. Plasma concentrations of LH and prolactin were not affected by diet on PND 160; however, mean plasma testosterone concentration was significantly higher in the E2 rats when compared to C2 rats. On PND 280, rats fed the E2 diet had significantly lower mean terminal body weights and absolute organ weights compared to the control rats. No other parameters were affected by diet at PND 280.

Authors' conclusion: A supplement of soy in the rat diet may affect growth and/or development of the reproductive tissues in male rats and also affect concentrations of reproductive hormones. The effects depend on the period of life when the soy diet is applied.

Strengths/Weaknesses: Dietary route of exposure, appropriate exposure period, and characterization of the diet are strengths. The lack of measurement of blood isoflavone levels and histopathology of testis, epididymis and prostate are weaknesses. Since LH and prolactin are variable endpoints, additional data would be useful to provide some understanding of the impact of the hormone changes on reproductive function. Number of exposed dams is unclear and litter effects were not taken into account.

Utility (Adequacy) for CERHR Evaluation Process: This study has limited utility for the evaluation process.

Mäkelä et al., 1990 ***(696)***, support not indicated, examined prostate effects in offspring of rats fed soy-containing or soy-free diets. From 1 week prior to mating through gestation and lactation, Sprague Dawley rats were fed a commercial rat diet containing 13% soy (control group, n=2 dams) or a soy-free, casein-based diet (n=3 dams). Pups were weaned at 21 days of age to the commercial (soy-containing) diet. Offspring were killed at 10 weeks of age, and prostates were removed and weighed in 13 or 14 animals/group. 5α-Reductase production was measured by determining levels of metabolites following incubation of prostates in media for 30 minutes. Prostates were then fixed in Bouin's fluid for sectioning and morphometric analysis. Data were analyzed by ANOVA followed by *t* test with Bonferroni correction. Prostate weights were significantly higher **[by ~40%]** in offspring of rats fed the soy-free diet. Diet had no detected effect on prostate 5α-reductase activity or volume density of epithelium, lumina, or intra-acinar stromal tissues.

Strengths/Weaknesses: Weaknesses of the study were the lack of comparison of chows, the small number of animals/group (n=2–3), and evaluation of only males.

Utility (Adequacy) for CERHR Evaluation Process: The study is of no utility for the present evaluation because of the small numbers of animals used and the limited number of endpoints examined.

McVey et al., 2004 ***(697)***, of Health Canada, reported serum and testicular androgen levels in F_1 rats exposed to a soy supplement added to the diet in a multigeneration study in Sprague Dawley rats. **[The full report of the multigeneration study is not yet available (G. Cooke, personal communication, December 2, 2004; CERHR asked for update on May 11, 2009 but none was provided]** Six diets were used in the study. The control diet was a soy-free casein-based diet (AIN93G) in which there were no detectable isoflavones by HPLC after β-glucuronidase digestion. A second diet was similar to the control diet but included a commercial soy-protein concentrate (Pro Fam 930) in place of casein. This diet was supplemented with a commercial soy-isoflavone concentrate (Novasoy®) at 4 different levels of supplementation. The total isoflavone levels in the soy-containing diets were 31.7, 36.1, 74.5, 235.8, and 1046.6 mg/kg feed **[ppm]**. Of the total isoflavones that were measured by HPLC after β-glucuronidase, 33–39% was daidzein, 8–10% was glycitein, and 52–59% was genistein. The authors noted that the range of isoflavone content in the diets represented a range of human consumption of soy isoflavones from the negligible amounts in North American diets to the high levels consumed by infants on soy formula. Diets were provided ad libitum from 50 days of age to male and female rats. After 70 days on the diets, animals were mated to produce an F_1 generation, which was weaned on PND 21 to the same diet assignment as the parents. F_1 males were killed on PND 28, 70, 120, 240, or 360 for measurement of testosterone and dihydrotestosterone in serum and in homogenized testes using a commercial ELISA kit. ANOVA with post hoc Tukey test was used for comparisons.

Based on feed consumption and body weight data, estimated F_1 total isoflavone intakes in the 6 diet groups at PND 28 were 0, 0.9, 1.2, 2.0, 6.3, and 29.5 mg/kg bw/day. On PND 120, estimated F_1 total isoflavone intakes were 0, 0.9, 1.0, 2.1, 7.3, and 28.7 mg/kg bw/day. There were no detected treatment-related alterations in mean body weight or feed consumption at any time during the study. Testis weights were increased by about 30% in the PND 28 males on the Novasoy®-containing diets

(total isoflavone intakes ≥2.0 mg/kg bw/day, n=5 or 6/treatment group). Serum testosterone peaked in all groups at the PND 70 measurement, consistent with puberty in rats, and remained elevated at PND 120 in the groups exposed to the diets with the 2 highest isoflavone levels. No differences between groups were detected at any time point other than PND 120 (n=12/group/time point except n=6/group on PND 360). Serum dihydrotestosterone followed the same pattern, with PND 120 levels statistically higher in the 2 highest isoflavone groups than in the other groups. No significant alteration in the ratio between testosterone and dihydrotestosterone was detected at any time point. Intra-testicular testosterone reached a peak on PND 120, at which time values in all soy-supplement groups appeared graphically higher than those in the control group but without evidence of dose-dependency and with a statistical difference from control only for the second-highest isoflavone-diet group. Intra-testicular dihydrotestosterone (ng/g testis) appeared to show a numerical decrease on PND 28 **[the only time point for which data were given]** as dietary isoflavone concentration increased, but no statistically significant changes were identified by the study authors. **[Trend test performed by CERHR was significant at *P*=0.015.]** The authors concluded that developmental exposure to isoflavones could alter testicular weight and androgen levels, although the mechanism for the apparent modulation of Leydig cell androgen production was not known.

Strengths/Weaknesses: Strengths of the study were determination of dietary isoflavone content, use of 5 different soy doses in chow, and monitoring of food consumption and soy intake. Weaknesses included examination of only males, lack of dose-response relationship for altered endpoints (e.g., plasma and/or testicular testosterone and dihydrotestosterone), lack of determination of reproductive capability, and lack of indication of the number of litters represented.

Utility (Adequacy) for CERHR Evaluation Process: This study is of limited utility based on the limited usefulness of the endpoints.

McVey et al., 2004 ***(698)***, support not indicated, examined the effects of lifetime exposure to isoflavones in the diet on steroidogenic enzyme activities in rat testes. Throughout gestation and lactation, female Sprague Dawley rats **[number not reported]** were fed one of six diets containing casein or soy as the protein source. The casein diet served as the control. The soy diets were alcohol-extracted to remove naturally occurring isoflavones and then fortified with Novasoy, an isoflavone supplement, at the isoflavone concentrations listed in **Table 142**. Male offspring were weaned on PND 21 and fed the same diets as their mothers. Male offspring (5–9/group) were killed on PND 28, 70, 120, 240, or 360. Enzyme activities were determined for 3β-hydroxysteroid dehydrogenase, CYP17, 17β-hydroxysteroid dehydrogenase, and 5α-reductase. Expression of messenger ribonucleic acid (mRNA) and protein was determined for 3β-hydroxysteroid dehydrogenase, CYP17, and P450 SCC, the mitochondrial cholesterol side chain cleavage enzyme involved in the first step of androgen production. Data were analyzed by linear and non-linear regression, ANOVA, and Tukey test. Enzymes activities declined with age in all dietary groups. As noted in **Table 142**, the only effects on enzyme activities occurred on PND 28 and included increased 3β-hydroxysteroid dehydrogenase activity in rats fed diets containing isoflavones 235.6 and 1046.6 mg/kg diet and increased CYP17 activity in rats fed diets containing isoflavones 235.6 mg/kg diet. No differences were detected compared to controls at later time periods or in other enzyme activities. There were also no detected differences in mRNA or protein expression. According to the study authors, these results suggest that isoflavones at levels consistent with infant exposures alter testicular enzyme activities in rats during development.

Table 142. Dietary Effects on Steroidogenic Enzyme Activities in Rats (McVey et al., 2004)

Protein Source[a]	Isoflavones, mg/kg feed (ppm)				Results
	Genistein	Daidzein	Glycitein	Total	
Casein	Not detected				Control diet
Soy	18.6	10.5	2.6	31.7	↔
	21	12.3	2.8	36.1	↔
	39.3	27.6	7.6	74.5	↔
	124.4	90.9	20.5	235.6	On PND 28: • ↑ 17β-HSD enzyme activity/testis • ↑ CYP17 enzyme activity/microsomal protein • ↑ CYP17 enzyme activity/testis
	544.8	412.3	89.5	1046.6	On PND 28: • ↑ 17β-HSD enzyme activity/microsomal protein • ↑ 17β-HSD enzyme activity/testis

↑, ↓, ↔ Statiscally significant increase, decrease, or no significant effect.

17β-HSD = 3β-Hydroxysteroid dehydrogenase.

[a] The soy-based diets were alcohol extracted to remove naturally-occurring isoflavones and then supplemented with varying amounts of isoflavones.

From McVey et al., 2004 *(698)*.

Strengths/Weaknesses: Strengths of this study includes the use of 5 different dose levels of soy, including doses with relevance to human exposure, and the measurement of isoflavone content of the diet. Weaknesses for this evaluation include the lifetime exposure to soy, the examination of only male offspring, the fairly small numbers of animals per group (n=5–9), and the lack of consideration of litter effects. The endpoints evaluated (steroid metabolizing enzyme activities and mRNA and protein expression) are of little utility alone, but may provide mechanistic information useful in interpreting results from other studies.

Utility (Adequacy) for CERHR Evaluation Process: This study is of limited utility based on inappropriate exposure time. It is also difficult to reconcile the results of this study with those reported earlier by this group using the same dietary conditions *(697)*. In their previous study, the authors demonstrated that serum testosterone and dihydrotestosterone were slightly (but not significantly) decreased at PND 28 compared to casein-exposed controls and were increased at PND 120. However, in this study, enzyme activities were altered at PND 28 but not at later time points, including PND 120. Additionally, the enzyme activity changes reported in this study were not dose-related.

Female and male rats

Hughes et al., 2004 ***(627)***, supported by EPA, conducted a study in rats to examine effects in female and male offspring of dams treated with soy milk during the lactation period. This study was conducted in Long Evans hooded rats that were fed a phytoestrogen-free AIN-93G diet in which the soy oil was replaced with corn oil. Dams were randomly assigned to groups given 2%-fat rice milk (controls, n=2) or 2%-fat soy milk (n=3) instead of water from PND 1 (day of delivery) to PND 21. The study authors estimated that intake of isoflavones through soy formula was 3.5–8.5 mg/day or 10–30 mg/kg bw/day. Pups had some direct exposure to the soy or rice milk once they were old enough to drink from the dam's bottle. Milks were replaced with water from PND 21 to the end of the study. Litter size was evaluated at birth, and pups were sexed, weighed, and evaluated for anogenital distance. Body weights and anogenital

distance were again measured at weaning. Pups were monitored for onset of puberty. Estrous cycles were monitored for 3 weeks in females. On PND 60, offspring were killed and male reproductive organs were weighed. A total of 14 male and 22 female pups exposed to soy milk and 8 male and 18 female pups exposed to rice milk were evaluated. Uteri from 6 offspring/group were fixed in 4% paraformaldehyde for histomorphometry examination and immunohistochemical analyses for proliferating cell nuclear antigen (PCNA), ERα, and progesterone receptor. Statistical analyses included ANOVA and Kruskal-Wallis test. The individual pups rather than the litter were considered the statistical unit. The pup-based analysis was said to have been used because intrauterine position of pups, which was not considered, was said to have a greater impact on variances of outcomes than differences between dams.

No effects of soy milk were detected on body weight, anogenital distance, or age of preputial separation in males, or on age of vaginal opening or estrous cycle length in females. Body weight was significantly higher and anogenital distance adjusted for body weight was significantly lower in female offspring of the soy group at weaning. Soy milk had no effect on relative (to body weight) weights of testis, seminal vesicles, or prostate in adult offspring. Relative epididymis weight was significantly reduced **[by 14%]** in the soy group compared to the rice-milk control. **[Absolute organ weights were not reported.]** The only significant effect noted in the uterus was a **[12%]** increase in progesterone receptor expression in glandular epithelial cells in females of the soy-milk group. Soy milk had no effect on uterine gland numbers, luminal epithelial cell height, uterine proliferation, ERα expression in luminal and glandular epithelial cells, or progesterone receptor expression in luminal epithelial cells. As discussed in other parts of the report, gestational and lactational exposure to genistein also increased expression of the progesterone receptor in uterine glandular epithelial cells. The study authors concluded that exposure of developing rats to isoflavones approximating human exposure levels induced an effect in an estrogen-responsive uterine marker long after cessation of exposure. Concerns were expressed because the progesterone receptor is involved in several reproductive processes.

Strengths/Weaknesses: Strengths of this study were use of a phytoestrogen-free chow, exposure during an appropriate period (lactation), exposure of rats to soy milk, and estimation of daily isoflavone intake. Weaknesses were the small numbers of litters used/group (2–3) and that the litter was not considered the experimental unit.

Utility (Adequacy) for CERHR Evaluation Process: This study is of no utility due to the small numbers of animals used.

Lund et al., 2001 ***(247)***, supported by the National Science Foundation and Brigham Young University, conducted a series of experiments to examine reproductive and developmental effects in Long Evans female and male rats exposed to phytoestrogen-containing feed during pregnancy, lactation and after weaning. The F_1 rats used in these studies were born to 5 F_0 dams/group that were fed throughout the gestation and lactation periods with a soy-based diet containing phytoestrogens 600 µg/g diet (phyto-600) or a phytoestrogen-free fish meal-based diet. The offspring were weaned on PND 30 to the same diets as their dams. Diets were similar in protein, fat, amino acid, mineral, and vitamin content. Data were analyzed by ANOVA followed by Bonferroni post hoc comparison.

In the first part of the experiment, female offspring (n=16/group) were monitored for day of vaginal opening and estrous cyclicity from 60 to 73 days of age. Body weights were measured in male and

female offspring at 50 days of age (n=20/sex/group). In the phyto-600 compared to phytoestrogen-free group, day of vaginal opening was significantly delayed (mean ± SEM=39.3 ± 0.5 days compared to 35.0 ± 0.3 days in the control). Diet had no effect on length or regularity of estrous cycles **[data were not shown]**. Body weights at 50 days of age were significantly lower in males and females fed the phyto-600 compared to the phytoestrogen-free diet **[by ~10% in females and ~6% in males]**.

In the second part of the study, 5–7 rats/sex/group given the phyto-600 diet continued to receive that diet, and 6 rats/sex/group were switched to the phytoestrogen-free diet at 80 days of age. Rats remained on each diet for 40 more days (until 120 days of age), at which time they were killed. Blood from 2 rats/group was pooled for measurement of serum phytoestrogen levels by GC-MS and measurement of 17β-estradiol and testosterone levels by radioimmunoassay (RIA). Body and prostate weights and volume of the sexually dimorphic nucleus of the pre-optic area (SDN-POA) were determined (n=5–7/sex/group). Body weights of male and female rats switched to the phytoestrogen-free diet were significantly higher than body weights of rats that remained on the phyto-600 diet **[7% higher in males and 13% higher in females]**. Males switched to the phytoestrogen-free diet also had higher absolute **[~ 21%]** and relative **[~ 15%]** prostate weights. Diet had no effect on serum testosterone or 17β-estradiol level in males. Males that remained on the phyto-600 diet had a significantly higher SDN-POA volume than males switched to the phytoestrogen-free diet. An increase in SDN-POA volume in females switched to the phytoestrogen-free diet compared to females remaining on the phyto-600 diet approached statistical significance. Total brain weight did not differ between dietary groups.

The study authors concluded that, "...phytoestrogens have considerable effects on hormonally sensitive somatic, reproductive organ, and neuroendocrine parameters. . ."

Strengths/Weaknesses: Strengths of this study were detailed descriptions of diets, measurement of serum phytoestrogens, and examination of appropriate endpoints. Weaknesses were comparison of only 1 phytoestrogen-containing chow to phytoestrogen-free chow, and the small number of animals used (n=5). Exposure through chow is a strength in its relevance to human exposure and a weakness in permitting only imprecise estimates of dose levels. In addition, the litter was not considered the experimental unit, and reproductive capability of animals was not determined.

Utility (Adequacy) for CERHR Evaluation Process: This study is of limited utility. The effect of switching the diet at 80 days of age is not relevant to developmental events.

Masutomi et al., 2004 ***(699)***, in a study supported in part by grants from the Japanese Ministry of Health, Labor, and Welfare, examined the effects of maternal or direct dietary consumption of a soy-free or soy protein containing diet on female and male offspring that were also exposed to ethinyl estradiol perinatally. Pregnant Sprague Dawley rats (n=15/group) were divided into groups fed CRF-1, a regular rodent diet containing soy proteins, or a soy-free diet. The CRF-1 diet contained genistin at 10.2 mg/100 g diet and daidzin at 8.7 mg/100 g diet. Soy-free diet was prepared according to the NIH-07 formulation, except that soy meal and oil were replaced with ground corn, wheat, and corn oil. **[It also appears that soy-free diets may have used fish or gluten meal as the source of protein.]** On GD 15 (day of vaginal plug=GD 0), 7 dams from each dietary group were given 0.5 ppm ethinyl estradiol through diet. Dosing was continued through PND 10 (day of delivery=PND 1). Pup anogenital distance was measured on PND 2. Five offspring/sex/group were necropsied on

PND 21 for measurement of organ weight. Eight offspring/sex/group were weaned to the CRF-1 diet and evaluated for onset of puberty. Estrous cyclicity was monitored from 8 to 11 weeks of age. Offspring were killed on PND 77–79, with females killed during diestrus. Organs were weighed and examined histologically. The litter was considered the statistical unit in evaluations conducted during the lactation period. For offspring data collected after weaning, individual animals were considered the statistical unit. Statistical analyses included ANOVA, Fisher exact test, and Mann Whitney U-test.

Because the purpose of this Expert Panel evaluation is to evaluate the effects of soy formula, discussion of results will focus on effects in groups fed the soy protein-containing versus soy-free diets and dietary effects on ethinyl estradiol responses. No effect of diet on maternal body weight gain during gestation or lactation was detected. No effect of diet on litter size was detected. On PND 2, male pups from the soy-free diet group weighed significantly less than males in the soy diet group. Offspring body weight gains on PND 10–12 in both sexes were significantly lower in the soy-free than the soy-diet group. Diet alone had no detected effect on absolute anogenital distance, but anogenital distance adjusted for body weight was slightly higher in females of the soy-free versus soy diet group. **[Diet was said to interact with ethinyl estradiol effects on anogenital distance in females, but the type of interaction was not clear.]** At the PND 21 necropsy, body weights of females in the soy-free groups were lower than in the soy diet group. Ovary weight relative to body weight was higher in the soy-free than the soy-diet group.

In offspring evaluated in adulthood, body weight gain on PND 21–42 was significantly reduced only in males from the ethinyl estradiol/soy-free diet group. Day of vaginal opening was significantly accelerated and body weight at vaginal opening was significantly lower in females from the ethinyl estradiol/soy-diet group. Body weight at preputial separation was significantly lower in the soy-free diet compared to the soy-diet group. Necropsies conducted in adult offspring revealed that the soy-free compared to the soy-protein diet significantly increased relative pituitary weights in males, decreased absolute and relative pituitary weights in females, and increased absolute and relative adrenal weights in males. Ethinyl estradiol induced a significant reduction in absolute and relative ovary weight only in offspring of rats fed the soy diet. The most prominent effects in female offspring exposed to ethinyl estradiol were irregular estrous cycles and histopathologic changes in reproductive tissues as outlined in **Table 143**. The number of rats with estrous cycle irregularities was statistically altered only in the soy-diet group, but the numbers were increased in both dietary groups exposed to ethinyl estradiol *in utero*. Incidence and severity of lesions in the ovary, uterus, vagina, mammary gland, and pituitary were greater in offspring of rats exposed to ethinyl estradiol and fed soy compared to soy-free diets.

The study authors concluded that typical estrogenic responses to ethinyl estradiol were enhanced by soybean-derived factors.

Strengths/Weaknesses: Strengths of the study were use of soy-free chow with similar nutritional contents as soy-containing chow, oral exposure route, determination of feed consumption, standardization of litter size (PND 10), and killing of adult females at the same stage of the estrous cycle. Phytoestrogens were measured in chow, but no methods and few data were presented. Weaknesses were the use of only 7 animals/treatment group, and lack of determination of reproductive capability.

Utility (Adequacy) for CERHR Evaluation Process: The study is of limited utility based on the small number of animals used.

Table 143. Adverse Effects in Rats Treated with Ethinyl Estradiol and Given Soy-Containing or Soy-Free Feed (Masutomi et al., 2004)

Parameter	*Ethinyl Estradiol, ppm*			
	0	*0.5*	*0*	*0.5*
	Soy Diet[a]		*Soy-Free Diet*[a]	
Irregular estrous cycles	0	8[c]	1	5
Persistent diestrus	0	0	0	3
Persistent estrous	0	6	0	1[d]
Increased ovarian follicles/decreased corpora lutea (±/+/++/+++)[b]	0	8 (0/1/0/7)[b]	0	3[d] (0/2/0/1)
Uterine epithelial hypertrophy (++/+++)	0	8[c] (3/5)	0	2[d] (0/2)
Endometrial squamous metaplasia (++/+++)	0	4 (3/1)	0	1 (0/1)
Vaginal mucosal hyperplasia	0	8[c]	0	1[d]
Vaginal diestrus mucosal change	8	0	8	6[d]
Mammary lobular hyperplasia (+/++/+++)	0	8[c] (1/6/1)	1 (1/0/0)	4[d] (3/1/0)
Anterior pituitary hyperplasia	0	8[c] (0/2/6)	0	3[d] (1/2/0)

[a] Values represent n affected of 8/group.
[b] Values in parenthesis are number of animals with ± minimal, + slight, ++ moderate and +++ severe effects.
[c] Statistically significant compared to same diet without ethinyl estradiol.
[d] Statistically significant compared to ethinyl estradiol group fed soy diet.
From Masutomi et al., 2004 *(699)*.

Odum et al., 2001 ***(622)***, supported by the Alkyl Phenol Ethoxylate Research Council, the Japanese Chemical Industries Association, and the Food Standards Agency **[a UK government agency]**, investigated the effects of different rodent diets during pregnancy, lactation and after weaning on sexual development in the female and male rats. Animals were Wistar-derived Alpk:APfSD rats. The diets included Rat and Mouse No. 3 (RM3), a standard diet in the UK for pregnancy and lactation, RM1, a standard diet for maintenance of rodents, Teklad Global 2016 (from Harlan Teklad), a natural diet made without soy or alfalfa, AIN-76A (from Harlan Teklad), a diet with sucrose, casein, and maize instead of soy and alfalfa, and Purina 5001 (from Purina Mills), a common US diet based on soy and alfalfa. Features of these diets are shown in **Table 144**. Diet combinations included a specified diet during pregnancy and lactation followed by the same or a different diet for weaned offspring. The pregnancy/weaning treatment diets were RM3/RM1, AIN-76A/AIN-76A, RM3/AIN-76A, Global 2016/Global 2016, and 5001/5001. Pregnant females were begun on their assigned diets on the day sperm were detected in the vaginal smear (GD 0). There were 12 females assigned to each of the 5 diet combinations. Genistein and daidzein content were measured in each diet by GC-MS. Females were maintained on the assigned diets through pregnancy and lactation. Offspring were maintained on their assigned diets until PND 68. From PND 70, offspring were all given the RM1 diet **[the diet between PND 68 and 70 was not specified]**. All females from 6 litters per treatment group were killed on PND 26, and sex organs were weighed. The remaining females were housed together by litter. Males were culled at weaning to 4 per litter (housed together). Developmental landmarks were

Table 144. The Five Diets Used by Odum et al., 2001

Component	Diets				
	RM1	RM3	AIN-76A	Global 2016	5001
Protein content, %	14.7	22.3	20.0	16.7	23.4
Energy, kJ/g	10.9	11.5	15.7	13.3	12.7
Soy content, %	6.0	13[a]	0	0	> 18[a]
Alfalfa content, %	0	0	0	0	3[a]
Genistein, ppm[b]	45	105	–	–	175
Daidzein, ppm[b]	30	70	–	–	110

– Negligible.
[a]Estimated by study authors.
[b]Estimated by CERHR from a graph in the paper.
From Odum et al., 2001 *(622)*.

monitored, including eye opening, testis descent, vaginal opening, and preputial separation. After vaginal opening, daily vaginal smears were taken until first estrus was identified. Daily vaginal smears were also taken on PND 52–69 to evaluate time spent in estrus. On PND 68, males from 6 litters per treatment group were killed and liver, kidney, and sex organs were weighed. On PND 68, theremaining females were culled to 4 per litter (housed together). The remaining males were killed on PND 128, and the remaining females were killed in estrus on PND 140–144 (after being on the RM1 diet since PND 70). Liver, kidneys, and sex organs were weighed. The RM3/RM1 diet combination was considered the control condition because this combination (for pregnancy/weaning) was used in most of the authors' previous studies. The 5001/5001 diet combination was also evaluated as a control diet, because it is used in many regulatory studies in the US. **[Analysis using the 5001 diet as a control was not shown in the paper.]** Statistical analysis was performed by ANOVA or ANCOVA (taking weaning weight as a covariate) with post hoc Student *t* test. The litter was the statistical unit. The proportion of animals recorded with a developmental landmark each day was evaluated by Fisher exact test.

Pregnancy outcome and offspring weight results are summarized in **Table 145**. **[Post-weaning food consumption and weight varied over the course of the experiment. The data graphs are difficult to interpret, but it appears that AIN-76A and Global 2016 may be suboptimal feeds for the post-weaning period in terms of body weight gain, feed consumption, or both. The authors note that the high-energy density of these diets may have influenced feed consumption. The authors imply that during the "recovery phase" after PND 70, when all animals were given RM1, feed consumption and weight tended to become similar among groups, although it did not reach parity in all groups.]** There were no effects of diet on eye opening. The effects of the diets on sex-related developmental landmarks are summarized in **Table 146**. Post-weaning exposure to AIN-76A appeared to consistently advance male and female pubertal landmarks compared to RM1, while the other 2 diets had less consistent effects. Effects on body weight and reproductive organ weight are summarized in **Table 146**. Absolute body weight and absolute organ weights were not evaluated for statistical differences; **Table 146** shows the effects of diet only with regard to body weight corrected for weaning body weight and with regard to relative organ weights. The authors noted that AIN-76A was associated with body weight gain that persisted during the recovery period, i.e., during the weeks following PND 70 when all animals were on the RM1 diet.

Table 145. Effects of Diet on Pregnancy and Lactation in Rats (Odum et al., 2001)

Parameter	Diet Combinations (Pregnancy/Weaning) Compared to RM3/RM1			
	AIN-76A/ AIN-76A	RM3/ AIN-76A	Global 2016/ Global 2016	5001/ 5001
Dam body weight				
Pregnancy	↔	↔	↔	↔
Lactation	↓3%[a]	↔	↓7%[a]	↔
Litter survival to weaning	"↑"	"↑"	↔	↔
Offspring weight[b]				
Birth	↓	↔	↔	↔
Weaning	↔	↔	↓	↓
Post-weaning	↑	↑	↔	↑

↑, ↓, ↔ Significant increase, decrease, or no change compared with RM3/RM1 diet.
"↑" indicates study author conclusion of an increase without statistical significance.
[a] Estimated from a graph.
[b] Results reported as suggested by the text of the paper. The data figures are not marked for significant differences.
From Odum et al., 2001 *(622)*.

In a second experiment, weanling female rats were obtained at 20–21 days of age. Animals were weaned on RM3 and then switched to RM1, AIN-76A, Global 2016, or 5001 (n=24/group) for 4 days. Half of the animals in each group received, respectively, 0 or 300 µg/kg bw/day sc of the GnRH antagonist antarelix for 3 days starting on PND 20 or 21. Animals were killed 24 hours after the last dose of antarelix. Body and uterine weights were compared with ANCOVA, considering initial body weight (for final body weight) and final body weight (for uterine weight) as covariates. The individual female was the statistical unit. Results are summarized in **Table 147** for final body weight (adjusted for initial body weight) and relative uterine weight. Absolute body and uterine weights were not analyzed statistically. Antarelix increased feed consumption by 24–40% **[data not shown]**.

The authors concluded that diet could affect sexual development, but they observed that the effects on sexual development were not consistent with presumed estrogenicity of diets like 5001 that are high in phytoestrogens. They noted, for example, that the greatest effect on sexual development appeared with post-weaning exposure to AIN-76A, a soy- and alfalfa-free diet with negligible phytoestrogen content.

Table 147. Effects of Diet on Relative Uterine Weight (Odum et al., 2001)

Endpoint	Diet and GnRH Antagonist (Antarelix) Dose, µg/kg bw/day							
	RM1		AIN-76A		Global 2016		5001	
	0	300	0	300	0	300	0	300
Body weight[a]	Control	↔	↑12%	↔	↔	↔	↑14%	↔
Relative uterine weight	Control	↔	↑50%	↔	↔	↔	↑68%	↔

↑, ↔ Significant increase or no change compared to control.
[a] Adjusted for initial body weight.
From Odum et al., 2001 *(622)*.

Table 146. Effects of Diet on Rat Developmental Landmarks and Reproductive Organ Weights (Odum et al., 2001)

Parameter	Diet Combinations (Pregnancy/Weaning) Compared to RM3/RM1			
	AIN-76A/ AIN-76A	RM3/ AIN-76A	Global 2016/ Global 2016	5001/ 5001
Testis descent				
Age	↑3%	↓3%	↔	↔
Body weight	↔	↔	↓8%	↔
Preputial separation				
Age	↓5%	↓7%	↔	↓4%
Body weight	↔	↔	↔	↔
Vaginal opening				
Age at onset	↓7%	↓10%	↔	↔
Body weight at completion	↔	↓12%	↓11%	↔
Age at first estrus	↔	↓11%	↔	↓8%
Body Weight (adjusted for weaning weight and relative organ weight)				
Females on PND 26				
Body weight	↑10%	↑11%	↓5%	↑14%
Uterus (blotted)	↑37%	↑83%	↑28%	↑77%
Uterus (dry)	↑30%	↑78%	↑24%	↑70%
Vagina	↑17%	↑42%	↑16%	↑27%
Cervix	↔	↑45%	↔	↔
Ovary	↔	↑11%	↔	↔
Males on PND 68				
Body weight	↑16%	↑9%	↔	↑13%
Liver	↑12%	↑10%	↔	↔
Kidney	↑7%	↑26%	↔	↑22%
Testis	↓10%	↔	↔	↔
Epididymis	↓9%	↔	↔	↔
Seminal vesicle	↔	↔	↔	↔
Prostate	↔	↔	↔	↔
Males on PND 128				
Body weight	↑10%	↑14%	↔	↔
Liver	↔	↔	↔	↔
Kidney	↔	↔	↔	↑9%
Testis	↓9%	↔	↔	↔
Epididymis	↔	↔	↔	↔
Seminal vesicle	↔	↔	↔	↔
Prostate	↔	↔	↔	↔
Females on PND 140–144				
Body weight	↑9%	↑13%	↔	↔
Liver	↔	↔	↔	↔
Kidney	↑22%	↑22%	↔	↔
Uterus (blotted)	↔	↔	↔	↔
Uterus (dry)	↓12%	↔	↔	↔
Vagina	↔	↔	↔	↔
Cervix	↔	↔	↔	↔
Ovary	↔	↔	↔	↔

↑, ↓, ↔ Significantly increased (older), decreased (younger), unchanged compared with RM3/RM1 diet.
From Odum et al., 2001 *(622)*.

They further noted that in the uterotropic assay, AIN-76A and 5001 produced an increase in relative uterine weight that was prevented with a GnRH antagonist, suggesting that the effects of diet involved alterations in hypothalamic function rather than simple estrogen exposure.

Strengths/Weaknesses: Strengths of the study were analysis of diet for genistein and daidzein content and analysis of appropriate endpoints. Weaknesses were differences in dietary protein and metabolizable energy content. Exposure through chow is a strength in its relevance to human exposure and a weakness in permitting only imprecise estimates of dose levels.

Utility (Adequacy) for CERHR Evaluation Process: This study is of limited utility based on the fairly large number of animals examined and the appropriateness of endpoints.

Pastuszewska et al., 2008 ***(700)***, supported by the National Committee of Scientific Research in Poland, examined the effects of the substitution of milk and egg for soy products in breeding diets for rats, with a concomitant decrease of the dietary protein level and supplementation with amino acids. Three diets were prepared: One diet contained soybean oil meal and soy concentrate as the main protein supplements (diet S), two diets (NS and NSA) were formulated without soy products **[source not stated, manufactured according to ISO 9001]**, the total protein was decreased and the soy protein was replaced by different amounts of whey protein concentrate and egg powder; the NSA diet was also supplemented by 1 g / kg DL-methionine (98%) and 2.2 g/kg of L-lysine (85%). Wheat middlings and sugar beet pulp were added to the NS and NSA diets to equilibrate the fiber content. Wistar rats (Ifz:BOA, 5- to 6-weeks old **[source not stated]**) were used on the study. *Assessment of the protein, energy and growth promoting values of the diets*: True digestibility (TD) and the biologic value (BV) of protein were determined on 25- to 26-day-old male rats (n=8/diet) fed 11g/day/animal for 10 days; feces and urine were collected quantitatively during the last 6 days. Net protein utilization (NPU) and content of available protein (NPV) were calculated. The metabolizable energy concentration was determined on 2.5- 3-month male rats (n=10/diet) fed the diets for 14 days; feces and urine were collected quantitatively during the last 7 days and the metabolizable energy was computed. Feed intake and growth rate were measured weekly in 22- to 23-day-old males (n=8/diet) fed the diets for 21 days. *Assessment of reproductive performance*: Seventy-five male and 75 female rats were assigned to one of the three diet groups, 25/ sex/group **[method not described]**. After 51 days, the animals were cohabited for 2 weeks (1 male: 1 female) within each group. On the day after parturition **[bedding not described]**, the number, gender and weight of each neonate (F_{1a}) was recorded and the litter size was reduced to 10 pups per litter. Pups were weaned at 22 days. Two weeks later, the F_0 males and females were again cohabited and F_{1b} litters were handled in the same manner. After weaning the second litter (F_{1b}), 12 dams from each group were randomly selected, killed, and organ weights were recorded. 12 F_{1b} males from the S group and the NS group were killed at weaning and the blood was analyzed for LH, prolactin, and testosterone. From the F_{1b} litters, 12 males and 12 females in each group (S, NS, and NSA) were selected for further rearing and fed the respective maternal diets. Body weights were measured at 36 and 60 days old. At 60-days-old, all F_{1b} rats were killed; hematologic and biochemical parameters were measured for all males, LH, prolactin, and testosterone were measured f or the S and NS group, and organ weights were recorded for the females. The results of one gender fed three diets were subjected to the one-way analysis of variance (ANOVA); those including both sexes were analyzed using the two-way ANOVA. The significance of differences in the hormone concentrations was determined by the non-parametric ANOVA rank Kruskal-Wallis test within each age group.

Table 148. Mating Efficiency in Rats on Soy-Containing Breeding Diet (Pastuszewska et al., 2008)

Mating	*S Diet (Soy-Containing)*	*NS Diet (Soy-Free)*	*NSA Diet (Soy-Free)*
First	0.88	0.88	1.00
Second	0.88	0.92	1.00

From Pastuszewska et al., 2008 *(700)*.

Feed intake and body weight gain was not affected during the first three weeks (F_0 generation) for either sex, or during the reproductive cycles for the F_0 females. Mating efficiency of the F_0 animals (delivered/mated) was affected by the diet regimen (**Table 148**); the two groups of animals fed the soy-free diets exhibited a greater mating efficiency than the animals fed a soy-based diet.

The number, the litter-based and the individual body weights of pups did not differ between groups in the first or second litters for neonates or weanlings. The effect of gender was significant on all neonate parameters for the first litters (F_{1a}) and on the mean body weight of the pups from the second litters (F_{1b}), but an interaction between diet and gender was not found. When the same parameters were analyzed jointly for both sexes and both litters, the number and the litter weights were both significantly higher on the NSA diet and tended to be higher on the NS than the S diet. Dam organ weights were not affected by diet. Growth rate of the F_{1b} offspring was not affected by diet. In the F_{1b} females at day 60, the mean liver weight was significantly higher in the animals fed the NSA diet, there were no other differences observed for F_{1b} female organ weights. In the F_{1b} males, there was no affect of diet on the red blood cell count and volume, hematocrit and hemoglobin concentration, or white blood cell count. The activity of hepatic enzymes was not affected and total cholesterol did not differ between groups. The hormone analysis of the S and NS groups was not different at 22 days of age; but at 60 days, LH and prolactin were considerably higher in animals fed S than NS diet, a difference in testosterone level was not confirmed statistically.

Authors' conclusion: Replacing soy protein by milk and egg protein with a concomitant lowering of the dietary protein level and amino acid supplementation does not impair the growth rate and tends to improve reproductive performance. However, feeding soy-free vs. soy-containing diets differentiates the hormonal status of young males.

Strengths/Weaknesses: Strengths of the study include evaluation of the effects of a soy containing diet versus two soy-free diets, information of compositions of diets were provided, determination of the metabolizable energy values of the diet, adequate numbers of animals were assigned to the reproductive performance study (25/sex/group for the F_0 generation), the F_0 animals were started on the diets 51 days prior to mating, food consumption was determined for the F_0 and F_1 generations, and 2 matings were conducted. Weaknesses of the study included the lack of analysis of the actual isoflavones in the soy containing diet, it is not clear if the litter was used as the experimental unit in the data analyses, and the majority of the data is represented as mean values with a pooled standard error which is not adequately clarified in the text. The article does not specify whether during culling to 10 pups/litter if there was an attempt to maintain an equal sex ratio where possible, the data for the mean number of pups weaned suggests that this was the case since the mean values vary between 4.1 to 5.0 for each sex. The criteria for considering a successful mating are not specified. Although

the calculated mating efficiencies were provided it would have been helpful to see the full data for mating performance i.e., number of females/group mated, number of females/group pregnant for each of the 2 cohabitation periods. The assumption is that there were 25 pairs cohabited/group for each cycle but this is not specified. Although plasma testosterone, LH, and prolactin were measured in the offspring at 22 and 60 days of age it would have been useful to have performed the same analyses on the F_0 males that were mated to be able to compare the effects of the different diets during adulthood versus during development. It is not clear why organ weights were only measured in females and the hematology and serum biochemical parameters were only determined in male rats. It would have been helpful to have evaluated these parameters in both sexes. Organ weights and histopathology in the male rats would have helped provide some context to the hormonal changes that were reported.

Utility (Adequacy) for CERHR Evaluation Process: This study alone has limited utility for the present evaluation. The data may be useful for comparison with other studies evaluating the effects of soy-free versus soy containing diets on growth and reproductive performance.

Raju et al., 2009 ***(701)*****,** supported by Health Canada, examined the effects of lifetime exposure to dietary soy isoflavones in an azoxymethane (AOM)-induced rat colon cancer model. Male and female Sprague-Dawley F_0 rats (Charles River Canada) were randomized into one of three groups to receive control, low, or high soy isoflavone supplemented diets. The control diet was an AIN-93G standard diet modified to contain alcohol-washed, isoflavone-free soy protein . The soy isoflavone-supplemented diets also contained (wt:wt) either low (40 mg) or high (1000 mg) doses of Novasoy-400 per kg diet. The total isoflavone content of Novasoy-400 was 40% **[basis of dose selection not stated]**. Male and female rats (F_0 generation) from the same treatment group were allowed to mate and deliver their litters **[initiation of exposure to the test diets not described] [bedding not described]**. F_1 pups were weaned on PND 27, the male pups remained on their parents diets until the end of the study (n=15 for control, n=20 for low soy isoflavone, and n=20 for high soy isoflavone); the female pups were used on a different study. At PND 45, the F_1 males received a subcutaneous injection of AOM, follow by a second injection one week later. 26 weeks after the first AOM injection the rats were killed and colons were dissected; macroscopic lesions and tumors were assessed and collected. Tumor incidence, percentage of animals with tumors, tumor multiplicity, mean tumor size, and tumor burden were calculated; ACF (aberrant crypt foci) were quantified. The following *in vitro* assays were conducted: cytotoxicity of soy isoflavones in DLD-1 cells and calculation of IC_{50} (the dose that causes 50% of the cells to undergo cytotoxic cell death), cell viability of DLD-1 cells treated with different concentrations of soy isoflavones, RNA isolation and RT-PCR analysis, and Western blot analysis. Significance was determined by one-way ANOVA with post hoc Tukey-Kramer test.

There were no differences between groups in behavior, food intake, weight gain, or terminal body weight; organ weights, hematology, and serum clinical chemistry were also unaffected by the levels of soy isoflavones in the diet. All rats developed ACF in their colon after treatment with AOM and the dose of soy isoflavones did not affect the mean number or size of ACF compared to the control group; total ACF did not differ between groups. All rats in the control and low soy isoflavone groups developed colon tumors, 95% of the rats in the high soy isoflavone group developed tumors. Tumor multiplicity did not differ between groups, mean tumor size per group and per tumor-bearing animal was significantly lower only in the low soy isoflavone group compared to controls, and tumor burden was lower in both soy isoflavone-treated groups. **[Reported effects did not show a dose response.]**

Necropsy revealed that the tumors were sessile polyps or polyploid growths that grew into the gut lumen and rested on the mucosa without a pedicle; no consistent tumor histotype could be attributed to any of the dietary groups, no soy isoflavone-related histological changes were discerned. Compared to control rats, the expression of ERβ mRNA relative to the β-actin gene was higher in tumors of rats receiving either level of soy isoflavones with no difference between the soy isoflavone-treated groups. DLD-1 cells responded to the cytotoxic action of soy in a dose-dependent manner; the calculated IC_{50} was 24.82 g/L; when cells were assessed for cell viability using trypan blue the calculated IC_{50} was 17.01 g/L. Compared to untreated cells, those treated with increasing concentrations of soy isoflavones (20-50 g/L) had higher levels of ERβ mRNA. At the protein level, cells treated with test doses of soy isoflavones had higher levels of ERβ than the control.

Authors' conclusion: The results suggest that pre- and postnatal exposure to dietary soy isoflavones suppresses the growth of colon tumors in male rats. The overexpression of ERβ in both rat colon tumors and DLD-1 cells caused by soy isoflavones suggests that ERβ is a critical mediator in mitigating its cancer-preventive effects.

Strengths/Weaknesses: Strengths of the study include the use of dietary exposure over the lifetime of the animal to isoflavones which is a relevant route of exposure, the use of a low and high soy isoflavone diet as compared to an isoflavone free soy protein diet, and the ratio of genistein, daidzein, and glycitein in the diet was specified (appeared to based on manufacturer information rather than actual analysis of the diets). Measurement of ERβ gene expression is a strength of the study. Weaknesses include that although food intake and bodyweights were measured during the exposure period the data are not provided so it is not possible to accurately calculate the doses of isoflavones administered, it is not clear as to how many litters per group were included in the study or how the 15-20 male pups were selected at weaning, it is unclear if the litter was considered as the experimental unit, the study was limited to evaluation of male pups, and there was no measurement of circulating isoflavone levels in the pups. Rational for the dose selection is not provided and is a weakness of the study. Moreover, the rational for the study is unclear. Specifically, it is unclear if the colon tumors are thought to be hormonally mediated and what role ERβ might have in the pathophysiology of colon tumors is uncertain. The use of *in vitro* model to assess the effect to genistein on cell viability using trypan blue exclusion provided limited mechanistic insight Use of chemical induction of colon tumors is a suitable model but its relevance to human colon tumor development is uncertain. The main weakness from the point of view of the CERHR evaluation is that the objective of the study was to assess the effect of dietary isoflavones on the growth of colon tumors and did not include endpoints that would have been useful to assess effects of the diets on development.

Utility (Adequacy) for CERHR Evaluation Process: The study has no utility to the present evaluation since the focus of the study was not on the evaluation of development. The data are interesting and may provide value in an assessment of the effects of diet on cancer incidence.

Ronis et al., 2009 ***(702)***, supported by USDA Current Research Information System, examined the effects of feeding soy or isoflavones on lipid homeostasis in early rat development; a second experiment examined the effects of feeding soy or isoflavones on the physiological, endocrine, and metabolic responses associated with consumption of a high-fat, high-cholesterol Western diet. Sprague-Dawley rats were obtained from Charles River **[bedding not described] [purity not stated**

for any compound] [basis of dose selection not stated]. *EXPERIMENT 1*: Pregnant rats were fed AIN-93G diet formulated with casein as the sole protein source (CAS diet) and allowed to deliver their litters. On PND 15, litters were assigned to one of five diet groups: Control (CAS diet), AIN-93G diet in which casein was replaced by soy protein isolates (SPI+ diet), AIN-93G diet in which casein was replaced by soy protein isolates that were stripped of phytochemicals (SPI- diet), AIN-93G containing casein and supplemented with 250 mg/kg genistein (C+G diet), and AIN-93G containing casein and supplemented with 250 mg/kg daidzein (C+D diet) **[number of animals/litter per group not stated]**. The SPI+ diet contained 286 mg/kg genistein and 226 mg/kg daidzein; it was estimated that pups fed the SPI+ diet consumed a mean of 80 mg of total isoflavones/kg BW/day. Pups were killed on PND 33, serum and liver were collected. Western immunoblot analysis was performed; Real-Time RT-PCR analysis of mRNA expression and ChIP analysis were conducted. **[Based on the Results section of the article, it appears that some animals from Experiment 1 may have continued on to at least PND 50, but this is not clear in the Methods, and it cannot be determined if these rats remained on their experimental diets or were returned to the control diet at some point.]** *EXPERIMENT 2*: Male rats received one of three diet regimens from PND 24 to 64, n=10 rats per group: the AIG-93G diet formulated with casein as the sole protein source (CAS diet), pair-fed a high-fat/high-cholesterol Western diet with casein as the sole protein source (Western casein diet), or pair-fed a high-fat/high-cholesterol Western diet with soy protein isolates as the sole protein source (Western SPI+). Body composition was assessed, and an oral glucose tolerance test (OGGT) was administered five days before killing (n=5 rats/group), and serum insulin concentrations were measured. The rats were killed, serum was collected, and liver and retroperitoneal fat pads were collected and weighed; serum insulin, glucose, triglycerides, and total cholesterol were measured. Liver sections were stained for lipid droplets using Oil red O, lipids were extracted from liver homogenates and triglycerides and cholesterol concentrations were assayed, and 16α-Hydroxylation of testosterone was measured in hepatic microsomes. In Experiment 1, data were analyzed by two-way ANOVA (diet X gender), in Experiment 2, data were analyzed by one-way ANOVA on ranks; differences among means were determined by the Student-Newman-Keuls test.

Serum genistein levels of male rats at PND 50 were higher in the C+G diet group (2700 nmol/L) than the SPI+ diet group (808 nmol/L). *EXPERIMENT 1*: Body weight at PND 33 was lower in males fed SPI+ or SPI- diets than the CAS diet (control), and serum IGF-1 concentrations were lower in males fed SPI+ or SPI- diets than the CAS diet (control). Expression and activity of male hepatic CYP2C11 were lower relative to the control group after both SPI+ and SPI- feeding. *PPARα-regulated pathways*: Expression of mRNA-encoding hepatic genes involved in peroxisomal mitochondrial fatty acid β-oxidation (ACO and HADHA) and fatty acid transport (CPT-1A) was greater in males and females fed the SPI+ diet than in rats fed the CAS diet. Feeding the SPI-, C+G or C+D diets resulted in no induction except for HADHA mRNA which was greater than controls only in female pups. All three genes were positively regulated by the transcription factor PPARα. Greater mitochondrial CPT-1A protein expression in the SPI+ diet group (but not SPI-, C+G, or C+D diet groups) was confirmed by Western immunoblot in livers of female rats. Expression of the mRNA encoding for PPARα itself was greater in both male and female rats fed the SPI+ diet compared to the CAS control diet. *PPARγ-regulated pathways*: Glucokinase and CD36 were expressed more highly in female than in male liver. Expression of both genes was greater after SPI+ diet and SPI- diet consumption in male rats compared with those fed the CAS control diet. Expression of these genes was not affected by the other diets and there was no effect of any diet on the female rats. In males fed the SPI+ diet,

ChIP analysis had greater PPARγ binding to its response element on the glucokinase promoter than the controls. PPARγ transcription factor expression (protein and mRNA) was also greater in nuclear extracts from male rats fed the SPI+ diet and the SPI- diet compared to the CAS control diet group. *LAXα-regulated pathways*: Expression of CYP7A1 and ABCG8 mRNA was greater after feeding the SPI+ diet than after feeding the CAS control diet in both sexes. ABCG5 was elevated only in the SPI+ diet-fed females. Greater expression of CYP7A1 after feeding the SPI+ diet was confirmed at the apoprotein level by Western immunoblot. Expression of all three mRNAs and of CYP7A1 apoprotein was greater in the C+G and C+D diet groups compared to the CAS control diet group for both sexes. ChIP analysis of LXRα binding to a response element identified in the CYP7A1 promoter had greater LXRα binding in the SPI+ diet group compared to control rats (both sexes). Feeding the SPI- diet did not affect LXRα binding. LXRα mRNA and protein expression was greater in nuclear extracts of the rats fed the SPI+ diet than in the CAS-fed male rats. *EXPERIMENT 2*: *Insulin sensitivity and lipid homeostasis*: Feeding the Western casein diet from PND 24 to PND 64 resulted in greater weight gain, percent body fat, and percent liver weight compared to the casein-fed control group. Feeding the Western SPI+ diet protected against the effects of fat and cholesterol on body composition. Analysis of OGTT after 33 days of feeding the Western casein diet resulted in a greater serum insulin concentration at 60 and 90 minutes post-glucose challenge compared with either the casein control or the Western SPI+ diet group. Insulin sensitivity appeared to be greater in the Western SPI+ diet group and was accompanied by a reversal of the greater serum glucose concentration in the Western casein group compared with the casein control group. Feeding the Western casein diet resulted in greater serum and hepatic triglyceride and cholesterol concentrations and in hepatic steatosis compared with casein-fed controls. Feeding the Western SPI+ diet resulted in reduced serum cholesterol and hepatic triglyceride and cholesterol concentrations and reduced steatosis when compared to feeding the Western casein diet. *Fatty acid synthesis and SREBP-1c activation*: Real-time RT-PCR analysis demonstrated no effects on SREBP-1c mRNA. Cleaved SREBP-1c protein was greater in the Western casein group than in the casein-fed control. Cleaved SREBP-1c was lower in nuclear extracts from the Western SPI+ group than in extracts from the Western casein group. Hepatic expression of the serine protease SKl-1/S1P was greater in the Western casein group than in the control; however, the expression did not differ between the Western SPI+ and the Western casein diet groups. mRNA for downstream gene targets of SREBP-1c controlling fatty acid synthesis and desaturation, FASN, and SCD-1 was greater after feeding the Western casein diet than the casein diet. Both these genes and another SREBP-1c target gene, ACC1, were lower in the Western SPI+ group than the Western casein group.

Authors' conclusion: The data demonstrate that feeding soy protein isolate-containing diets to prepubertal rats resulted in increased expression of hepatic genes regulated by the nuclear receptors PPARα, PPARγ, and LXRα and decreased expression of genes regulated by SREBP-1c. These effects may partially explain the antisteatotic, cholesterol-lowering, and insulin-sensitizing effects of soy.

Strengths/Weaknesses: Strengths of the study include the use of dietary exposure, culling was used in experiment 1 to standardize the size of the litters, and the use of various endpoints to assess the effect of soy or isoflavones on cholesterol homeostasis. A weakness of the study is a lack of comprehensive information on food and isoflavone intake, or on circulating isoflavone levels. In addition the diet contents were not adequately described so it is not clear what the nutritional value was for each of the diets used and how this might have influenced body weight gain. Serum genistein levels were

measured in male rats at PND 50 fed the SPI and the C+G diets which was helpful, however, there is no description of the analysis method, the number of rats included in the analysis, or the a description of the exposure period. The assumption is that the rats were on the diets from PND 15 to 50. For experiment 1 is not clear how many litters were treated or how animals were selected for generation of samples for measurement of biochemical, mRNA, and protein endpoints.

Utility (Adequacy) for CERHR Evaluation Process: The study has limited utility for the present evaluation due to the lack of experimental detail. The data suggest that dietary soy protein has effects on various molecular endpoints involved in PPAR and LXR signaling pathways which may have beneficial effects on circulating cholesterol levels.

3.4.1.2 Mice

Badger et al., 2008 *(703)*, supported by the USDA-ARC, examined the effects of feeding soy protein isolate rather than genistein in the diet of *A^{vy}/a* mice as well as the effects of soy protein isolate on body composition and hepatosteatosis in Sprague-Dawley rats of two ages . Diets were prepared using the published AIN-93G formula except that soy oil was replaced with corn oil; two experimental diets were prepared: 100% of the protein as casein, or 100% of the protein as soy protein isolate. Three experiments were conducted. *Experiment #1 (yellow A^{vy}/a mice)*: The breeding colony was derived from a highly inbred VY/WffC3Hf/Nctr-A^{vy} colony at the National Center for Toxicological Research/FDA, Jefferson AR. Black *a/a* dams were bred to mottled yellow and pseudoagouti *A^{vy}/a* sires to produce *A^{vy}/a* and *a/a* offspring for this study. Breeding pairs **[number of animals not stated]** were fed one of the experimental diets two weeks prior to mating and through gestation and lactation **[bedding not described]**. At four weeks of age, the offspring were weaned onto the diets of their parents **[number of offspring not stated]** and coat color was categorized: black, pseudoagouti (PAG), almost pseudoagouti (APAG), heavily mottled yellow (HMY), mottled yellow (MY), slightly mottled yellow (SMY) or clear yellow (CY). Offspring were killed when they were 75 days old; blood, liver, fat pads and skin were collected. The percentage of mottling was determined for the entire skins of the *A^{vy}/a* mice; liver sections were stained with Oil-Red-O and quantified by MCID imaging for hepatosteatosis. Hepatic gene expression profiles were determined for male *A^{vy}/a* MY/SMY/CY mice in each group (n=3/group). *Experiment #2 (PPARα signaling)*: Pregnant Sprague-Dawley female rats (Harlan, Indianapolis, IN) were placed on one of the experimental diets on gestation day 4 **[number of animals not stated] [bedding not described]**. Offspring were weaned onto the same diet as their dams and were sacrificed before puberty on PND 34; hepatic Acyl Co-A Oxidase (ACO) levels were determined and binding of PPARα to the ACO promoter assessed. *Experiment #3 (overfeeding-induced hepatosteatosis)*: In order to produce obesity, adult Sprague-Dawley male rats (Harlan, Indianapolis, IN) were surgically cannulated with an intragastric tube and fed high fat diets (45% corn oil + 0.5% cholesterol) for 21 days at 115% of the National Research Council recommended caloric needs of adult rats **[number of animals not stated]**. The diets were prepared with either casein or soy protein isolates as the sole protein source. After 21 days the males were killed and hepatic steatosis was determined by Oil-Red-O staining. Total lipids were extracted from the liver homogenates and triglycerides and cholesterol concentrations were determined. *All experiments*: Student *t* test was used to compare data from two groups. For multiple group comparisons, data were analyzed by one-way or two-way ANOVA with post hoc Student-Newman-Keuls tests. Linear regression was used to quantify the relationship between body composition variables and the coat color (% black).

Experiment #1: No differences in coat color distribution were observed between diet groups. Body weights and fat pad weight in male soy protein isolate-fed mice were slightly lower than the casein-fed males, but this was not statistically significant; diet did not affect body or fat pad weights in female mice. *A^{vy}/a* mice with the MY/SMY/CY phenotypes had hepatosteatosis, whereas the lean APAG and PAG phenotypes had no obvious ectopic fat deposition. *A^{vy}/a* SMY mice fed the soy protein isolate diet had lower hepatosteatosis as compared to the casein-fed SMY and CY offspring. Forty-nine gene transcripts were altered by soy protein isolate feeding in livers of male *A^{vy}/a* SMY/CY mice. Gene ontology analyses revealed that the majority of genes affected by consumption of soy protein isolates had important catalytic activities or gene binding properties; approximately 10% of the genes also had a known role in regulating gene transcription and a greater gene expression (4.19-fold) of CYP4a14 was observed. Analyses suggest a greater expression of four genes regulated by SREB-1C; however, neither SREB-1c mRNA nor the classic lipogenic targets (e.g., fatty acid synthase or malic enzyme 1) were higher. *Experiment #2*: Real time RT-PCR analysis of liver from casein- and soy protein isolate-fed rats demonstrated greater ACO mRNA; hepatic nuclear extracts from rats fed the soy protein isolate diet had greater binding to PPRE. Real time RT-PCR analysis demonstrated greater expression of mRNA encoding for PPARα itself in the soy protein isolate-fed rats compared to the casein-fed rats. *Experiment #3*: Neither body weight gain nor body composition differed significantly between diet groups. Hepatosteatosis developed in the high fat-casein-fed group and levels of hepatosteatosis were 30% lower in the soy protein isolate-fed rats; liver triglyceride and cholesterol content was also reduced for the soy protein isolate-fed mice.

Authors' conclusion: The effects of purified genistein differ from those of soy protein isolate when genistein equivalents are closely matched. Soy protein isolate does not epigenetically regulate the agouti locus to shift the coat color phenotype in the same fashion as genistein alone and soy protein isolate may be beneficial in management of non-alcoholic fatty liver disease.

Strengths/Weaknesses: Strengths of the study include the use of dietary exposure, soy-free diet, and exposure during gestation and lactation which are critical periods of development. The study lacks detailed information on animal numbers, and a dose-response was not assessed. When animal numbers are presented in Figures and Tables, the largest number or animals per group did not exceed 12. Table 2 in the paper displays the effects of SPI on hepatic gene expression (from 3 mice). Fold changes represent ratios of mean gene expression of SPI group compared to CAS group. Fold changes of +/-1.5-fold changes are deemed statistically significantly different via a Student's t test. All of the fold changes displayed are statistically significant. There are questions on the statistical significance because the comparisons are clearly not independent and the significance level is not being adjusted for the large number of comparisons being made.

The composition of the diets and isoflavone content were not analyzed so the potentially active ingredients in the diets were not defined. In addition, food consumption and body weight data were not provided, and circulating levels of isoflavones were not determined so it difficult to estimate the amounts of soy-protein isolate that the mice were exposed to (which would explain why a dose-response analysis was not conducted). Because the two diets differed in soy and casein content an alternative hypothesis to the author's conclusion is that there is something about the casein diet that increases fat in liver. The line of agouti mice used in this work is not the same as those used by Jirtle and colleagues. The lack of color change in the soy protein fed group might be due to a difference in the methylation sites on the two agouti (Avy) genes. Moreover as the authors point out the addition

of Gen to a casein based diet is different from a soy protein diet without casein. They also point out that their diet contains equol which may have affected their results. Equol is only processed by about 25% of adults and no infants, as such is not a major soy protein for human health concerns. It was not clear if the litter was used as the experimental unit in the analyses. This manuscript did not state the number of animals at the inception of the study nor the number of offspring (animals per litter). Because the experimental design is not adequately described it is difficult to evaluate the results of this study because of the varying sample sizes used in the reported results

Utility (Adequacy) for CERHR Evaluation Process: This study has no utility for the present evaluation.

Guerrero-Bosagna et al., 2008 ***(704)***, supported by FONDECYT, CONICYT, MECESUP and NH&MRC, examined the effects of a continuous pre-and post-natal exposure to high levels of dietary isoflavones on sexual maturity, morphometric parameters and DNA methylation status in mice. Adult male and female C3H mice **[source not stated]** were assigned to one of two experimental treatments: control diet **[feed not described] but standard laboratory chow typically contains soy** , or control diet plus a commercial concentrate of soy isoflavones (Soy Life®) added at 2% (ISF diet) **[purity of the soy isoflavone concentrate not stated]**. The proportion of soy isoflavone was based on a previous rat study. Total isoflavones were about 5 times higher in the ISF diet as compared with the control. Treatment was initiated two weeks prior to mating and mice were allowed to deliver their litters (n=13 litters for control diet group, n=12 litters for the ISF diet group) **[bedding not described]**. **[The duration of treatment is not stated for the F_0 generation, but appears to be through lactation. The diet fed to the pups is not stated, but it appears that the pups were fed a control diet after weaning.]** Pup body weights (g), size (cm), and ano-genital distances (mm) were measured on PND 7, 14, 21 and 42 (n=24 for control diet, n=19 for ISF diet). Beginning PND 20, all female pups were checked daily for vaginal opening (n=39 for control diet, n=32 for ISF diet). The Student's *t* test or two-way ANOVA (diet and gender) were used as the statistical tests. Offspring were killed after PND 42 and DNA was obtained from the pancreas and liver. DNA methylation patterns in skeletal α-actin (*Acta1*) (n=3-5/sex/group); ERα and *c-fos* were measured (n=4).

There were no differences in pup sex ratio or litter size between the control diet and the ISF diet groups. Sexual maturation was advanced by approximately 6 days in female pups born to the soy isoflavone-fed mothers; vaginal opening occurred at 31.6 PND for the control diet group and 25.7 PND for the ISF diet group; body weight, size and ano-genital distance in females at the day of vaginal opening were lower in the ISF diet group compared to the control diet group. There were no differences in size or weight at PND 7, PND 14, or PND 21 between the groups; however, the pups in the ISF diet group were lower in size and weight on PND 42. There was no difference in the ano-genital distance between groups for males or for females.

For *Acta1* in liver, MANOVA showed no differences in DNA methylation between pups born to mothers fed the ISF but sex differences were noted in liver from control diet feed animals. Thus the isoflavone diet suppressed the sex differences. For *Acta1* in pancreas, MANOVA revealed no gender differences in the control or ISF diet groups and no differences were observed between experimental groups within males or females; however pooling male and female data together revealed an effect of diet on 2 of the 8 methylation sites. For ERα promoter in liver, MANOVA showed no gender differences in either group and no multivariate effects of treatment were detected. A lack of methylation across the promoter of *c-fos* was observed in the liver and pancreas for both groups.

Authors' conclusion: The data demonstrate that a diet rich in phytoestrogen can result in advancement of sexual maturation in female pups as well as suppress normal gender differences in the DNA methylation pattern of a tissue-specific methylated gene such as *Acta1*. These results support the hypothesis that alterations in the hormonal state of the pregnant females produced by a diet of phytoestrogens or other xenoestrogens can affect phenotype as well as the epigenetic state of the offspring.

Strengths/Weaknesses: Strengths include that soy/isoflavones (doses determined in previous study) were given orally and there were adequate animal numbers (12-13 litters/treatment). A commercial mixture of soy isoflavones was used which is good since humans consume soy as mixtures, but also a limitation since it is harder to know which compound is active. DNA methylation was used as an endpoint for epigenetic effects. A weakness is that the study did not appear to control for litter effect.

Utility (Adequacy) for CERHR Evaluation Process: The study provides limited utility for the evaluation process. The study but does suggest that the isoflavone diet affects onset of puberty DNA methylation as an interesting end point for epigenetic effects provides some mechanistic information. The two obvious target genes, ERα and cfos were not affected by the diet, but Acta1 was. Actin is a ubiquitous component of all tissues and thus this shows that soy diet could have large and general actions.

Mäkelä et al., 1995 ***(305)***, supported by Turku University Foundation, Yrjö Jahnsson Foundation, and Emil Aaltonen Foundation, evaluated the anti-estrogenic effects of lifetime exposure to a soy diet in developing male Han-NMRI mice. Animals were given either a standard laboratory chow containing 7% roasted soybean meal or a commercial soy-free feed. Female mice were fed either the soy diet or the soy-free diet during pregnancy and lactation **[n not given]**. At weaning, half the males in each litter were continued on their dams' diet and half were switched to the opposite diet (n=27–29/group). At 2 months of age, urethroprostatic blocks were harvested for dissection of seminal vesicles, coagulating gland, ventral prostate, and dorsolateral prostate. Relative weights of these organs and testes were recorded, and histopathology was evaluated on the reproductive organs of 5 animals/group. An additional experiment was performed using males exposed during pregnancy, lactation, and thereafter to 1 of the 2 diets (15–19/group) with harvesting and examination of reproductive organs at 12 months of age. Other experiments described in this paper used the same design (exposure during pregnancy, lactation, and thereafter), but also treated male pups with sc diethylstilbestrol on the first 3 days of life. Evaluations of the reproductive organs of these males were performed at 2, 9, or 12 months of age (n=13–19/group). Statistical analysis used the Student-Newman-Keuls multiple-range test, Fisher exact test, or ANOVA with post hoc *t* test. **[The number of animals in the data tables were lower than the number of animals identified in the Methods section for some experiments.]**

Coagulating gland and combined prostate lobe relative weights were increased in 2-month-old males fed a soy diet after weaning, following a soy-free diet given to the dam during pregnancy and lactation, when compared to animals exposed to soy during pregnancy and lactation and after weaning. The relative weight of the prostate lobes was also significantly higher than the comparable organ weights of males placed on a soy-free diet after weaning, regardless of which diet their dams had been fed. The increase in prostatic lobe relative weight in the group fed soy after being weaned from soy-free dams was 11–19%. There were no detected body weight changes or accessory gland histologic alterations associated with soy feeding status. When males were evaluated at 12 months after exposure to the same soy-free or

soy-containing diets as their dams received during pregnancy and lactation, relative reproductive organ weights were increased by soy feeding. The magnitude of the increase was 40% for ventral prostate, 60% for coagulating gland, 63% for dorsal prostate, 35% for seminal vesicles, and 20% for testes. No body weight changes or histological differences associated with soy feeding status were detected. In neonatally estrogenized males evaluated at 2 months of age, soy feeding of dams and offspring was associated with a 46% increase in relative weight of the coagulating gland and an 81% increase in relative weight of dorsolateral prostate, without a change in body weight. There was a soy-associated decrease in the proportion of animals with severe dysplasia when the accessory reproductive organs were histologically evaluated at 9 months of age (3/10 compared to 8/10). The authors also stated that the incidence of severe dysplasia was decreased by soy feeding at 12 months, although the difference between groups was not statistically significant (7/11 compared to 12/14 **[*P*=0.76, Fisher exact test]**).

The authors concluded that there was an anti-estrogenic effect of feeding a soy diet during male development because of amelioration of diethylstilbestrol-associated prostatic growth inhibition and because of a delay in diethylstilbestrol-associated prostatic dysplasia. There was no conclusion concerning the differential effects of soy exposure of dams during pregnancy and lactation or soy exposure of offspring after weaning.

Strengths/Weaknesses: A strength was that the 2 diets were analyzed and found to be similar in protein, vitamin, and mineral content. In addition, urinary isoflavones and lignans were measured. Weaknesses were lack of indication of the number of animals/group and apparent lack of consideration of the litter as experimental unit. Exposure through chow is a strength in its relevance to humans and a weakness in permitting only imprecise estimates of dose levels.

Utility (Adequacy) for CERHR Evaluation Process: The study is of limited utility based on lack of experimental details.

Robertson et al., 2002 ***(705)***, supported by the National Health and Medical Research Council of Australia, examined the effects of a soy-based diet on male aromatase-knockout mice. The knockout mice, which are unable to synthesize endogenous estrogens, are initially fertile, but at around 18 weeks of age, spermiogenesis is disrupted as a result of a post-meiotic defect. Groups of wild-type and aromatase-knockout mice were fed a diet that contained 10% soy meal or a soy-free diet. Total isoflavone levels were reported at 146 mg/g soy-based diet. The study authors stated that each type of diet was fed to mice beginning at birth. **[It is not clear if the diets were fed to offspring and parents. Most likely, the parental mice were exposed because the authors stated that all knockout mice were fed the soy-free diet for 2 years prior to the study, with the exception of 10 mating pairs fed the soy-containing diet.]** One set of wild-type and knockout mice (n=6–12/group) fed either the soy-free or soy-containing diet and exposed during the same time period were killed at 14 weeks of age. A second set of wild-type and knockout mice fed the soy-free diet (n=11–12/group) were killed at 1 year of age, and data obtained from these mice were compared with data obtained from mice fed the soy-containing diet in a previous study (n=6–8/group). **[The Expert Panel notes that 1-year-old animals from the two different diet groups were not exposed concurrently.]** Parameters examined included terminal body weight, testis weight, testicular morphology, spermatogenic germ cells in the testis, and serum LH and FSH levels. Statistical analyses included ANOVA and least-squares significant difference test.

Terminal body weights were significantly higher in 14-week-old wild-type **[24% higher]** and knockout mice **[17%]** and in 1-year-old knockout **[25%]** mice fed the soy-free versus soy-containing diet. Testis weight was not affected by diet or genotype. Qualitative evaluations of testes revealed more pronounced disruption of spermatogenesis in 1-year-old mice fed the soy-free versus soy-containing diet **[data not shown].** Quantitative testicular observations that were statistically significant are summarized in **Table 149**. Few and minimal effects were observed in 14-week-old mice. The study authors noted that at 1 year of age, knockout mice fed the soy-containing diet had increased spermatogenic cell numbers compared to knockout mice fed the soy-free diets. Compared to knockout mice fed soy-free diet, the knockout mice fed the soy-containing diet had significantly lower **[40%]** serum FSH levels, but no significant effects of dietary exposure on LH levels in knockout mice were detected. The study authors concluded that low levels of dietary phytoestrogens exert biological effects on the testis that are independent of effects on the pituitary-gonadal axis.

Table 149. Summary of Significant Effects in Wild-Type and Aromatase-Knockout Mice Fed Diets With and Without Soy (Robertson et al., 2002)

Parameter	*Genotype and Dietary Comparisons*[a]			
	KO-Soy$^+$ vs. KO-Soy$^-$	*KO-Soy$^+$ s. WT- Soy$^+$*	*KO-Soy$^-$ vs. WT- Soy$^-$*	*WT-Soy$^+$ vs. WT-Soy$^-$*
14 Weeks of Age				
Seminiferous tubule diameter	↑13%	↔	↔	↑8.3%
Seminiferous tubule length	↓12%	↔	↔	↓13%
Testicular spermatogonia numbers	↓29%	↓29%	↔	↔
Testicular round spermatid numbers	↑22%	↔	↔	↔
Sertoli cell numbers	↔	↑7.7%	↔	↓7.1%
1 Year of Age				
Seminiferous tubule lumen volume	↑550%	↔	↓80%	↔
Testicular epithelial volume	↑160%	↓32%	↓79%	↓21%
Testicular interstial volume	↔	↔	↑109%	↑45%
Seminiferous tubule diameter	↑31%	↔	↓33%	↔
Seminiferous tubule length	↔	↔	↓50%	↓14%
Testicular spermatocyte numbers	↑317%	↔	↓81%	↔
Testicular round spermatid numbers	↑360%	↓54%	↓90%	↔
Testicular elongated spermatid numbers	↑400%	↓56%	↓92%	↔
Total germ cell numbers/Sertoli cell numbers	↑240%	↓55%	↓86%	↔

[a] Percent changes were estimated from a graph by CERHR.
KO, aromatase-knockout mice; WT, wild-type mice; Soy$^+$, soy-containing diet; Soy$^-$, soy-free diet.
↑,↓,↔ Significantly increased, decreased or no change.
From Robertson et al., 2002 *(705)*.

Strengths/Weaknesses: Strengths are that chow was analyzed for isoflavone content, the study used reasonably sized groups (n=6–12), and the use of appropriate endpoints to evaluate the testis including detailed morphometry. The lifetime exposure to soy and the use of only 1 dose level are weaknesses.

Utility (Adequacy) for CERHR Evaluation Process: This study is of no utility based on the inappropriate exposure period and use of only a single dose level of soy. However, this study did demonstrate that estrogen is required for normal spermatogenesis and that the phytoestrogens in soy can partially compensate for the inability to synthesize estrogen in aromatase-knockout mice.

Ruhlen et al., 2008 ***(706)***, supported by grants from the National Institute of Environmental Health Sciences (NIEHS), the Kapor Foundation, the W. Alton Jones Foundation, and National Institutes of Health (NIH), examined the reproductive and metabolic characteristics in male and female mice reared and maintained on non-soy low-phytoestrogen feed or soy-based high-phytoestrogen feed. Adult CD-1 mice (*Mus musculus domesticus*), from CRL (Wilmington, MA) were housed with corncob bedding. There were seven different experiments conducted on this study. *General Methods*: Adult female mice were randomly selected to be placed on either PMI 5K96 or PMI5008 chow, they were paired with males and allowed to deliver their litters; F_0 females remained on their respective diets throughout pregnancy and lactation. The number, weight and sex of F_1 pups was recorded on the day of birth. At weaning on postnatal day (PND) 20, pups that had been reared on PMI 5008 were switched to PMI 5001 maintenance chow - the pups reared on PMI 5K96 remained on this feed after weaning. The PMI 5K96 feed does not contain soy (casein is one of the sources of protein in this feed); PMI 5008 and PMI 5001 feeds are soy-based and thus contain soy isoflavones. For postnatal studies, 18 pups were assigned to the PMI 5008/5001 diet and 19 were assigned to the PMI 5K96 diet. All organ weight data were first analyzed by analysis of covariance (ANCOVA) to determine whether a correction for body weight was needed; data were then reanalyzed by analysis of variance (ANOVA). If overall ANOVA or ANCOVA was statistically significant, planned comparisons using the LS means test was conducted. Results were considered statistically significant at $P<0.05$. *Experiment #1 (estrogenic activity of the feed)*: Estrogenic activity in the feed was measured by methanol extraction and an examination of the degree to which the extract stimulated proliferation of Human MCF-7 breast cancer cells was conducted. *Experiment #2 (estradiol in pregnant females and fetuses)*: 13 F_0 females fed PMI 5K96 and 10 F_0 females fed PMI 5008 were killed on gestation day 18 and the fetuses were delivered. Maternal and fetal blood was collected for measurement of serum estradiol levels. Fetal blood was pooled within each litter and sex - thus each litter yielded one value for males and one value for females. *Experiment #3 (postnatal body weight, abdominal fat, and serum leptin)*: One randomly-selected male and female from each litter was weighed at birth, at weaning and in adulthood. On PND 90, the gonadal and renal fat pads were examined as a measure of obesity. Blood was collected for measurement of serum leptin. *Experiment #4 (adult glucose tolerance)*: The animals from experiment #3 were examined one week earlier for fasted glucose tolerance. *Experiment #5 (uterine response to estradiol and serum leptin in prepubertal females)*: Two randomly-selected females per litter were weaned on PND 17. Females were implanted with capsules containing either 0.25 μg 17β-estradiol (this dose results in ~80% of the maximum uterine growth response that can be induced with estradiol at this age) or the vehicle (stripped corn oil). Uteri were examined on PND 20. *Experiment #6 (body weight, uterine weight, histology and serum leptin on PND 26)*: 10 F_1 females from each diet group were weaned and weighed on PND 19; on PND 26 (~puberty), the females were killed and blood was collected for analysis of serum leptin. Body weights and uterine weights were recorded, and the uterus was saved for histologic analysis of luminal epithelial cell height. *Experiment #7 (onset of fertility in females)*: Two randomly-selected females per litter were weaned on PND 20, paired with sexually experienced males and monitored for the age at parturition. The number, body weights and sex ratio of the pups was recorded. *Experiment #8 (adult male reproductive organs)*: On PND 90, reproductive organs were weighed and collected from F_1 male mice fed PMI 5K96 (n=24) or PMI 5008/5001 (n=22).

Experiment #1 (estrogenic activity of the feed): Low estrogenic activity for the batch of PMI 5K96 feed (expressed as genistein equivalent units; 3.9 ppm) was low compared to the batches of PMI 5008 (40.0 ppm) and 5001 (25.8 ppm). *Experiment #2 (estradiol in pregnant females and fetuses)*: Serum estradiol levels in fetuses whose mothers were fed PMI 5K96 feed were significantly higher than levels in the fetuses from mothers maintained on PMI 5008. There were no differences in serum estradiol levels in the pregnant females fed the PMI 5008 or PMI 5K96 feeds. *Experiment #3 (postnatal body weight, abdominal fat, and serum leptin)*: Pups produced by PMI 5008-fed females were significantly heavier at birth than pups produced by PMI 5K96-fed females; there was no effect on body weight at weaning (PND 20); on PND 90, the PMI 5K96-fed males and females were significantly heavier than PMI 5008/5001 (males were 11% heavier, females were 27% heavier). Gonadal and renal fat pads from animals fed PMI 5K96 weighed more than those fed PMI 5008/5001 (93% and 115% more, respectively; for males; 126% and 86% more, respectively, for females). Serum leptin was 121% higher in males and 174% higher in females fed PMI 5K96 compared to males and females fed PMI 5008/5001. *Experiment #4 (adult glucose tolerance)*: Basal blood glucose levels and glucose clearance after a glucose injection were not different based on type of feed in females; males fed PMI 5K96 had impaired glucose clearance compared with males fed PMI 5008/5001. *Experiment #5 (uterine response to estradiol and serum leptin in prepubertal females)*: On PND 20, uterine weights in females with the corn oil capsule did not differ based on diet. Uteri of females fed PMI 5K96 were significantly heavier in response to estradiol stimulation compared to those of the females fed PMI 5008/5001. Neither feed nor estradiol treatment influenced serum leptin levels. *Experiment #6 (body weight, uterine weight, histology and serum leptin on PND 26)*: There was no effect of diet on body weights at weaning. On PND 26, females fed PMI 5K96 were significantly heavier and had significantly larger uteri, greater epithelial cell height, and significantly higher serum leptin. *Experiment #7 (onset of fertility in females)*: The females fed PMI 5K96 produced pups at a significantly younger age (44.7 days) than the females fed PMI 5008/5001 (46.6 days). There was no significant difference in the mean pup weight or the number of pups produced by females on either diet. *Experiment #8 (adult male reproductive organs)*: The testes, epididymides, seminal vesicles, liver and right kidney were significantly smaller in males fed PMI 5K96 than in males fed PMI 5008/5001. The prostate was heavier in males fed PMI 5K96 than in males fed PMI 5008/5001.

Authors' conclusion: Removing phytoestrogens from mouse feed produces an obese phenotype consistent with metabolic syndrome, and the associated reproductive systems are consistent with Fetal Estrogenization Syndrome (FES) due to elevated endogenous fetal estradiol. Laboratory rodents have become adapted to high-phytoestrogen intake over many generations of being fed soy-based commercial feed; removing all phytoestrogens from the feed leads to alterations that could disrupt many types of biomedical research.

Strengths/Weaknesses: Strengths of the study include dietary exposure, control for litter, the number of animals appears adequate, and study is thorough with multiple endpoints. Weaknesses of the study include the lack of characterization of the active ingredients administered to the animals, estrogenicity of the diet was defined using the MCF_7 assay and stimulation expressed as genistein equivalent units. However, there was no measurement of plasma isoflavones. The conclusions by the authors are overstated and more data are needed to support concluding statements.

Utility (Adequacy) for CERHR Evaluation Process: This study has limited utility for the present evaluation. The data are useful for the assessment of effects of soy versus soy-deficient diets on body weight, body fat, and leptins.

3.4.1.3 Monkeys

Anthony et al., 1996 *(707)*, funding not indicated, presented a limited examination of hormonal levels and reproductive organ weights in rhesus monkeys (*Macaca mulatta*) fed soy protein diets as part of a study examining the effects of soy isoflavones on cardiovascular risk. Peripubertal male (1.3–2.1 years old) and female (1.3–4.1 years old) monkeys were fed diets containing casein and lactalbumin as the source of protein for 3 weeks. The monkeys were then stratified randomly according to age and cholesterol levels. For a 24-week period, 1 group of 7 females and 5 males was fed a soy-based diet containing 1.27 mg genistein and 0.42 mg daidzein per g soy protein. The diet resulted in isoflavone intake of 9.41 mg/kg bw. **[Assuming that genistein and daidzein were the only isoflavones present, intake of each respective isoflavone would be ~7 and 2 mg/kg bw.]** A second group of 7 females and 6 males was fed a diet in which the soy protein was alcohol-extracted to remove most of the isoflavones. Levels of isoflavones in the isoflavone-reduced diet were 0.121 mg genistein and 0.052 mg daidzein per g soy protein. The reduced-isoflavone diet provided an isoflavone intake of 0.97 mg/kg bw **[genistein ~0.7 mg/kg bw and daidzein ~0.3 mg/kg bw]**. As part of the cross-over study design, each group of monkeys received 1 of the diets for 24 weeks and was then switched to the opposite diet for 24 weeks. Blood levels of 17β-estradiol, testosterone, dehydroepiandrosterone sulfate, and thyroxine were measured by RIA, and sex hormone-binding globulin was measured by a saturation assay. It appears that results were pooled for animals of the same treatment groups regardless of exposure period. Following the second exposure period, monkeys were killed and prostate, testis, and uterus weights were obtained. Data were analyzed by ANCOVA and paired *t tests*. The presence of dietary isoflavones in the soy diet had no detected effect on hormone levels or reproductive organ weights. The authors reported the intact isoflavone protein had favorable effects on plasma lipid and lipoprotein concentrations. **[As noted by study authors, all male monkeys were prepubertal at the start of the study and some reached sexual maturity during the study. Results could have been affected by imbalances in male sexual maturity.]**

Strengths/Weaknesses: A strength of this study was use of rhesus monkeys, which is presumably one of the most relevant species for humans. The cross-over design allowed for measurements from the same animal on both diets (soy-based and alcohol-extracted), which is valuable given the small sample sizes. Monkeys were randomized based on age and the ratio of total plasma cholesterol to HDL cholesterol, one of the primary confounders in examining the soy effects on plasma lipid and lipoprotein concentrations. Diets were kept frozen until needed, which may have improved test material stability. Levels of genistein and daidzein were determined in both the soy-based and alcohol-extracted soy protein. Proteins were also analyzed for the presence of β-sitosterol. Samples were analyzed in duplicate. Statistical analyses seemed appropriate with analyses for main effects of treatment and period as well as any interaction between those terms. Some weaknesses were also noted. As is often the case in studies using monkeys, this study contained small sample sizes (n=14 females and 13 males at study start). Results were pooled for all animals receiving the same diet, regardless of differences in age or other factors. After the second 24-week exposure, animals were killed and reproductive organ weights were measured. It was assumed that the endpoints measured reflected the last diet to which the animals were exposed (i.e., that there were no long-term effects of soy-based diet because both groups had received this diet prior to necropsy). It was not possible to

control for differences in the state of sexual maturity of the animals at the beginning versus the end of the study, making it unclear whether differences in hormone measurements or reproductive organ weights were masked. Data on these parameters and their variances were not provided. Furthermore, there was no evidence that the authors controlled for diurnal variation in their measurements of hormone levels. Despite attempts to control dietary components, the authors noted that "differences in the concentration of the protein in the products somewhat affected the percent of energy as protein, fat and carbohydrate," among other factors that could influence study outcome.

Utility (Adequacy) for CERHR Evaluation Process: This report is of no utility in the evaluation.

Sharpe et al., 2002 ***(708)***, support not indicated, fed male marmoset offspring a soy infant formula (Wysoy®) or a cow-milk formula (SMA Gold™). The animals included 13 pairs of co-twins assigned to different formulas to permit paired statistical tests and 4 additional animals. At 4–5 days of age, the marmoset offspring were intermittently separated from their mothers by a wire mesh divider, permitting mothers and young to be within sight of one another. The periods of separation included the 8-hour work-day on weekdays and about 2 hours on weekends. During this time, the infants remained with their fathers and siblings in the family cage. Because marmoset young typically receive care (other than feeding) from their fathers and older siblings, these periods of separation did not appear to distress either the infants or the mothers. During the periods of separation, infants were hand-fed their assigned formulas using a soft tube connected to a syringe. The formulas were prepared according to the instructions for human infant feeding and were administered in quantities limited only by the marmoset infant's appetite. Animals were formula-fed 3 or 4 times on weekdays and once or twice on weekend days. Except for periods of separation, the marmoset infants were caged with their mothers and permitted to nurse. Based on the composition of the formulas and the amount consumed, the investigators estimated that the marmoset infant intake of isoflavones during the 5–6 weeks of the study was 1.6–3.5 mg/kg bw/day. On PND 18–20 and 35–45, infant blood was taken for plasma testosterone determination by ELISA. In 7 pairs of twins, the second blood sample was obtained by cardiac puncture after the animals were killed for evaluation. The earlier blood sample in these animals and both blood samples in the animals that remained alive were obtained from the femoral vein. Testes and pituitaries were removed from the animals killed on PND 35–45. Testes were weighed after fixation in Bouin fluid. Testes were sectioned and evaluated for Sertoli and germ cell number, Leydig cell number per testis using immunostaining for 3β-hydroxysteroid dehydrogenase, and intensity of staining for 17α-hydroxylase/C_{17-20} lyase. Pituitaries were fixed in Bouin fluid, sectioned, and immunostained for the β-subunits of LH and FSH. Statistical comparisons were made using paired *t* tests.

No differences were detected between treatment groups in body weight at the beginning or the end of the treatment period. No difference in formula intake were detected between the treatment groups. Plasma testosterone was similar between the treatment groups on PND 18–20, but was lower in the soy formula-fed group on PND 35–45 when analysis was restricted to the 13 pairs of twins (mean ± SD 1.3 ± 2.1 ng/mL compared to 2.8 ± 3.9 ng/mL in the cow-milk formula-fed group, P=0.004 by paired *t* test on log-transformed data). The proportion of 35–45-day-old animals with plasma testosterone < 0.5 ng/mL was 1/15 in the group given cow-milk formula and 12/15 in the group given soy formula (P<0.001, Fisher exact test). Testis weights were comparable between the 2 groups, as were Sertoli and germ cell numbers/testis. Leydig cells/testis were increased 74% in the soy formula-fed group at 35–45 days of age. No differences by treatment group were detected in intensity of staining for

17α-hydroxylase/$C_{17–20}$ lyase in testis sections and numbers of cells immunopositive for β-subunit LH or FSH in pituitary sections. Because blood concentrations of LH and FSH cannot be reliably measured in the marmoset, the authors could not determine whether the decrease in plasma testosterone was due to an effect of soy constituents on the pituitary or on the Leydig cell, but they believed the decrease in plasma testosterone to be potentially important, particularly in light of the normal increase in testosterone that occurs in neonatal primates, including humans.

Strengths/Weaknesses: Strengths of this study included use of a non-human primate species, appropriate exposure period, exposure to soy formula, and adequate numbers of animals/group. A weakness is that because animals were allowed to nurse, total soy exposure was unknown. Specifically, maternal chow was not described and possible exposure occurring through mother's milk was unknown. In addition, only one soy formula dose was used.

Utility (Adequacy) for CERHR Evaluation Process: The study is of limited utility based on appropriateness of exposure period and species used. The endpoints are of limited utility alone in determining reproductive effects, but may be helpful in interpreting results from other studies.

Tan et al., 2006 *(709)*, [support not indicated] examined the effects on puberty progression, reproductive function and testicular cell numbers in adult male marmoset monkeys (*Callithrix jachus)* that had been fed soy formula as infants. **[No information provided post-weaning feed.]** The study was not conducted according to GLP, but was conducted according to the Animal Scientific Procedures (UK) Act 1986. The animals were captive-bred common marmoset monkeys (*Callithrix jacchus*). A total of 14 newborn marmosets were followed in this study: seven sets of co-twins (assumed fraternal). One twin from each set was fed SMA (cow milk-based formula) as the control; the other twin was fed SFM (soy formula milk). Both formulae were manufactured by SMA Nutrition and were purchased from the supermarket as a powder, diluted with tap water and heated according to manufacturer's instructions. No additional analysis was conducted, citing published literature for expected levels of aglycone and isoflavones in this brand of formula. Beginning on day 4 or 5 **[not defined]** and continuing through day 35 to 45 (five to six weeks), each infant was isolated from his mother for approximately 8 hours (weekdays) or approximately 2 hours (weekends) and left with his father. The appropriate formula was offered to the infant via a syringe fitted with a soft rubber tube and the infant was allowed to drink until he decided to stop drinking. Infants were fed 3 or 4 times on weekdays, and once or twice on weekends. For the remainder of the day, the mothers nursed the infants. Based on the actual measured intake, this regimen is believed to have exposed the infants to an isoflavone intake of between 40% and 87% of the reported intake for human infants aged 4 months who were fed a 100% SFM diet. Serum testosterone levels were measured at 10-week intervals from 40 weeks of age through 100 weeks of age and again at 120 weeks. At 80-104 weeks, each male was cohabited with a female and maintained in cohabitation for 24-80 weeks until the end of the study **[It is not clear how long animals were cohabited. Animals aged 80-104 weeks could not have been cohabited for 80 weeks and necropsied at 138 weeks of age]**. Pregnancy was confirmed by palpitation and the number of offspring was recorded. **[No formal assessment of sexual or mating behavior was conducted.]** At the age of 120 to 138 weeks, all males were killed, terminal body weights were recorded and a necropsy performed. **[Necropsy procedures were not described; necropsy data were not presented.]** The testes with epididymides, prostate, seminal vesicles, pituitary, thymus and spleen were fixed in Bouin's solution and then weighed; the stretched penis length was recorded. The right testis was imbedded in JB4 resin for enumeration of Sertoli cells and the left testis was imbedded in paraffin,

examined histologically and used to determine Leydig cell numbers. A paired *t* test comparison of body weight, testis weight and cell number/volume was conducted. Testosterone data was log-transformed before the *t* test was performed. **[Serum levels of isoflavones were not determined.]**

There was no effect of SFM formula compared to SMA formula on terminal body weight, necropsy observations, or the following organ weights: prostrate, seminal vesicle, pituitary, spleen, thymus; there was also no effect on stretched penis length. The onset of puberty, as gauged by serum testosterone levels, was not affected by SFM feeding vs. SMA feeding; there was no significant difference in fertility or serum testosterone levels between the two feeding groups at any age. The mean testicular weight, number of Sertoli cells (7%), and the number of Leydig cells (32%) was consistently and statistically significantly higher in the SFM-fed group. The mean seminiferous epithelial volume and the mean seminiferous tubule lumen volume were slightly higher in the SFM-fed males compared to the SMA-fed males, but there was no consistent trend between co-twins and the difference was not statistically significant.

Authors' conclusion: Infant feeding with SFM has no dramatic adverse reproductive effects in male marmosets, although it alters testis size and cell composition, and there is consistent, if indirect, evidence for possible compensated Leydig cell failure. Study results do not rule out the possibility that infant feeding with SFM might induce adverse effects in small proportion of exposed males.

Strengths/Weaknesses: Strengths of the study include the use of a co-twin design to minimize the number of animals used and to control for litter effect and large inter-animal variability, and maintenance of family groups during treatment to minimize stress. Another strength of the study is that marmosets were exposed orally to infant soy formula during the neonatal period with feeding on demand, which is very relevant to human infants. In addition, the isoflavone intake of soy-formula fed marmosets was estimated to allow comparison with human intake. The study used a non-human primates, a highly relevant animal model to humans. When determining Leydig cell numbers, the authors based the number of fields counted per animal on obtaining a percentage standard error value of <5%, which is a strength, as is reporting the detection level and intra- and inter-assay coefficients of variation for testosterone assays. When determining testosterone levels, sample from co-twins were processed at the same time. The decrease in plasma/serum testosterone levels and increase in Leydig cell numbers with soy formula treatment agreed with results seen at 35–40 days of age in soy formula-fed marmosets in the previous Sharpe *et al.*, 2002 study *(708)*. A weakness of the study design includes the nursing of the marmosets by their mothers which complicates the assessment. Marmosets were returned to their biological mother for 16 hours/day on weekdays and 22 hours/day on weekends; thus, breast feeding during this interval may have masked some effects of an entirely soy-based diet. Other weaknesses include the lack of characterization of the diets of the mothers and their isoflavone content, there were no data on blood levels of genistein or daidzein. In addition, there was no assessment of a dose-response and time-to-mating was not calculated.

Utility (Adequacy) for CERHR Evaluation Process: This study has limited utility for the evaluation process. The observations of no adverse reproductive effects on male marmosets are useful data especially since they were generated in a non-human primate model.

Wagner et al., 2009 ***(710)***, supported by the National Center for Research Resources, examined the differences in lipids, glycemic measures and body weight in the adult female cynomolgus monkey

and their offspring when fed a diet with the protein derived from soy or a diet with the protein derived from casein-lactalbumin. The monkeys used in this study were part of a National Center for Research Resources-supported cynomolgus monkey (*Macaca fascicularis*) breeding colony. 19 adult females were followed, during that time 25 offspring were born (12 males and 13 females). Adults (mean age 5.3 years) were initially fed Monkey Diet 5037 (Purina LabDiet®), a low fat, low cholesterol, high carbohydrate diet with the protein derived from soy. After one year on this baseline diet they were assigned to one of two breeding groups. Both groups had their diet changed to approximate the typical American diet (TAD) with 35% of calories from fat, a moderate amount of cholesterol and less carbohydrate than the standard monkey chow. One group was fed the TAD diet with the protein derived from soy (TAD soy, n=6) and the other group was fed the TAD diet with the protein derived from casein-lactalbumin (TAD casein, n=11). Offspring remained in the natal groups with their mothers during lactation; after weaning they continued to eat the TAD diet fed to their mothers. The TAD soy diet provided an isoflavone aglycone-equivalent dose of 180 mg/monkey/day. The isoflavone component present in the TAD soy diet consists of approximately 40-50% genistein, 40% daidzein and 10-15% glycitein. The mixture is the naturally occurring ratio of isoflavones in soy products and the monkeys consumed 3-4 times the Japanese median daily isoflavone intake. The TAD casein diet was nearly devoid of soy and soy isoflavones. Blood samples and body weights were obtained from the adults during the baseline and after consuming the TAD diets. Total cholesterol (TC), HDL cholesterol (HDLC), triglyceride (TG), glucose, plasma apoB-containing lipoprotein cholesterol (apoB-C), insulin and fructosamine concentrations were determined. An intravenous glucose tolerance test (GTT) was done in adult females after consuming the TAD diets for at least 1 year and in offspring that were, on average, 24 months of age (n=12 in the TAD casein group, n=13 in the TAD soy group). Blood samples for serum isoflavone concentrations were determined in 17 adult females 4 hours after a meal during baseline (consuming chow) and again after changing to the TAD casein or TAD soy diets. Serum isoflavones were also determined in 18 of the 25 offspring 4 hours after a meal. Analyses were either *t tests* or ANOVA. Tests of heterogeneity of variance were performed for all analyses and all variables that failed were log-transformed.

In both adults and their offspring, the TAD soy diet resulted in significantly higher serum isoflavone concentration than the TAD casein diet. Offspring consuming TAD casein had similar isoflavone concentrations to adults consuming TAD casein, but offspring consuming TAD soy had higher concentrations than the adult females consuming TAD soy. In this study, serum isoflavone concentrations during chow feeding were higher than when adults were consuming TAD. Equol made up the majority of the isoflavones. In adults, there were no differences in body weight, TC, TG, apoB-C, and glucose between monkeys fed TAD casein and monkeys fed TAD soy. Fructosamine concentrations were significantly lower in the monkeys fed TAD soy compared to monkeys fed TAD casein. There were no differences in HDLC or insulin concentrations between the two TAD groups. In the offspring, there were no differences in body weights at birth; but by one year of age and continuing to two years of age, offspring consuming TAD casein weighed significantly more than those eating TAD soy. These offspring also had impaired glycemic control as evidenced by higher fructosamine concentrations, but there was no difference in fasting glucose and insulin concentration, however there was a significant difference between the sexes: females showed higher insulin in the TAD casein group, while males showed higher insulin in the TAD soy group. HDLC was higher in females than males, but there were no differences in TC, HDLC, or apoB-C with diet; TG concentrations, however, were lower with TAD soy than TAD casein. Responses to the GTT were not different with TAD diet

in adult females. In the offspring, the glucose AUC was significantly lower and the disappearance of glucose was significantly faster with TAD soy than TAD casein; the insulin responses to the glucose challenge were also significantly lower in the TAD soy group. The glucose AUC was significantly higher in females compared to males, but there was no diet X sex interaction.

Authors' conclusion: Early nutrition may play a role in obesity and related conditions. The use of commercial chows rich in soy isoflavones adds an uncontrolled variable to many studies. As most primate resources feed standard monkey chow, this could result in a very different disease pattern than people in Western countries who eat very limited amounts of soy protein.

Strengths/Weaknesses: Strengths of the study include use of dietary exposure, the contents of the diets were provided, and information was included on the levels of genistein, glycitein, and total isoflavones in the diets as measured by the supplier. In addition, serum isoflavone levels were measured in the mothers and the offspring which is a strength of the study, although it is not clear from the experimental details as to when these analyses were performed which is a weakness. The offspring were exposed during critical periods of development i.e., during gestation, lactation and through infant life either via the mother or directly from the diet. The study was focused on evaluation of lipid measurements and glycemic control but was not designed to evaluate parameters relative to reproduction and development.

Utility (Adequacy) for CERHR Evaluation Process: The study has limited utility for the evaluation of developmental effects. However, this could be a useful colony of animals to evaluate the consequences of lifelong exposure to a soy diet on lipid profile and glycemic control.

3.4.1.4 Other Species

Cardosa and Bao *(711)*, [funding not stated], investigated the effects of chronic consumption of a soy meal-containing diet or soy isoflavones supplement on the morphology of reproductive organs, semen quality, age that males reached puberty, and sexual behavior of male rabbits. Sixteen female New Zealand rabbits - approximately 8 months old - were randomly assigned to one of 4 treatment groups: Group 1 (Control group) was fed a soy and alfalfa free diet (S-) and dosed daily with a cornstarch placebo; Group 2 was fed a soy and alfalfa free diet plus 5 mg/kg bw/day of soy isoflavones (ISF_05); Group 3 was fed a soy and alfalfa free diet plus 20 mg/kg bw/day of soy isoflavones (ISF_20); Group 4 was fed a diet containing 18% soy meal plus a cornstarch placebo (S+), this diet provided about 13 MG/KG BW/DAY of isoflavones. The cornstarch placebo or isoflavones (40% pure) was inserted directly into the oral cavity. **[the day F_0 dams began treatment was not stated - it appears that they were placed on the treatment regimen prior to mating].** The females were all mated with the same male and allowed to deliver their litter. **[bedding was not described]** When the pups were 5 weeks old they were weaned and 10 male pups per group were placed on the same treatment regime as their mothers. From 100 to 170 days of age, semen samples were collected once weekly, after this initial period the semen was collected every other day for 5 weeks (17 total collections). The first 7 collections were to stabilize sperm output and not analyzed, the last 10 ejaculations were used to quantify: ejaculate volume, sperm motility (1-100%), vigor evaluation (0-5), sperm concentration, and sperm morphology (% abnormal). Age at puberty was established when the semen reached the following characteristics concurrently: sperm concentration over 75 X 10^6 sperm/ml, motility over 50%, and vigor of 2.5 or higher. Sexual behavior was assessed using: time of reaction (latency to begin mounting), the interval between two ejaculations, and mounting reflex. At 230 $\pm$ 3 days of age, males were killed and

the testes, epididymides, proprostrate and prostate glands were weighed and fixed in Bouin's; slides were prepared and stained with H&E prior to examination for abnormalities. Body weight and food consumption were measured weekly throughout the study for all animals. Comparisons between S+ and S- (Control) groups were performed using analysis of variance (ANOVA). Comparisons among ISF_05, ISF_20 and S- (Control) groups were made using ANOVA ($P < 0.05$), followed by Tukey's test.

The sexual maturation of the bucks occurred at ages compatible with breed standards - however the data showed that soy meal-dietary treatment of rabbits (S+) resulted in more precocious males compared to the Controls (S-). There were no significant differences among the S-, ISF_05, and ISF_20 groups. Rabbits in the ISF_20 group weighed significantly less than the Control group beginning at 13 weeks of age, food consumption was significantly reduced for this group beginning at 17 weeks of age. There were no differences in organ weights between any of the treatment groups. There were no histopathological differences in the testes or the accessory sex glands for any treatment group. There were no significant differences in the age that the males exhibited the mounting reflex or in the time of reaction between the Controls and any other treatment group. The interval between two consecutive ejaculations was less ($P < 0.01$) in the ISF_20 group when compared to the ISF_05 and the Control group, but there was no difference between the S+ and the S- groups.

Authors' conclusion: Soy meal as a main source of protein in the diet of rabbits does not induce deleterious effects on sexual behavior or semen production. Also, normal dietary treatments with soy isoflavones did not cause toxic effects on the evaluated variables of males. However, this effect cannot be extrapolated to females which are more sensitive to the effect of environmental estrogens.

Strengths/Weaknesses: Strengths of the study include dietary exposure, use of 2 dose levels of isoflavones, the number of animals assessed, and inclusion of multiple endpoints relevant to the assessment of the male reproductive tract. The use of rabbits rather than rodents for male reproduction studies allows a longitudinal assessment of semen parameters. The lack of information on the treatment period for the F_0 dams, lack of detail on the morphological assessment of the testis, and the lack of measurement of isoflavone exposures in the animals are weaknesses. Litter effects were not taken into account.

Utility (Adequacy) for CERHR Evaluation Process: This study has limited utility for the evaluation. The lack of effects of soy on semen parameters are useful data.

Cardoso and Báo *(712)*, [support not indicated], examined the effects of perinatal (intrauterine and lactational) exposure to soy-containing diet and soy-derived isoflavones on the reproductive parameters of males rabbits. Pregnant does **[strain and source not stated, number of does not stated, bedding not described]** were randomly assigned to one of four groups: 1) control diet (a soy- and alfalfa-free diet), 2) control diet supplemented with 10 mg/kg bw/day of soy isoflavones, 3) control diet supplemented with 20 mg/kg bw/day isoflavones, or 4) a diet containing 18% soy meal **[it appears that the isoflavone content of the soy isoflavone used in diets 2 and 3 was 40%, but this is not clear; the isoflavone content of the soy meal used in diet 4 is not stated]**. The animals in the control group receive cornstarch as a placebo. The proper dose of isoflavones (or placebo) was placed directly in the mouth of the rabbit **[gestation day at the beginning of treatment not stated, basis of dose selection not stated]**. The diets contained similar levels of nutrients, but the protein source used in the control and isoflavone diets was cottonseed meal and meat meal; the soy-containing diet

is a commercially available diet. Eight male pups from each group were weighed and killed between 29 and 31 days of age. Testes and epididymides were weighed and the left testis and epididymis was processed for light microscopy; the diameter of the seminiferous tubules and epididymal duct were measured. The remaining males were weaned on day 35 and placed on the control diet until the end of the study. Between 15 and 21 weeks of age, the rabbits were allowed to mount a mature doe and the semen was collected using an artificial vagina. The time of reaction (latency to begin mounting), the interval between two consecutive ejaculations, and the mounting reflex were determined. At 26 weeks of age, males were subject to one semen collection every other day for 5 days (3 collections); ejaculate volume, sperm motility, vigor evaluation, and sperm concentration were recorded. At 27 weeks of age, the males were weighed and killed, the following organs were weighed and prepared for light microscopy: testes, epididymides, proprostate, and prostate. Different parameters were analyzed using analysis of variance (ANOVA).

Soy-based diet and consumption of soy-derived isoflavone intake during gestation and lactation did not affect dam feed intake, duration of gestation, or litter size. In male offspring, at 30 $\pm$ 1 days of age, there was no effect of treatment on body weight or on the histopathology of the testes or epididymides. There was no evidence of feminization or demasculinization of the reproductive tract in the male progeny of any treatment group; organ weights were comparable between all groups and histopathological examination revealed no changes attributed to treatment. Male sexual behavior was unaffected by treatment, and there were no significant differences in ejaculate volume, sperm motility, vigor, or concentration.

Authors' conclusion: The results indicate that intrauterine and lactational exposure to soy-containing diet and soy-derived isoflavones may not adversely affect reproductive development and function in male rabbits.

Strengths/Weaknesses: Strengths of the study include dietary exposure, 2 dose levels of isoflavones, and inclusion of multiple endpoints relevant to the assessment of the male reproductive tract. The lack of experimental detail such as definition of the diet contents and description of the morphological assessment of the testes and epididymides, and the lack of measurement of isoflavone exposures in the animals are weaknesses.

Utility (Adequacy) for CERHR Evaluation Process: This study has limited utility for the evaluation process.

3.4.2 Mammary Gland Development and Carcinogenesis

Hakkak et al., 2000 ***(713)***, funded by the USDA, examined the effects of diet containing soy or whey protein on the development of dimethylbenzanthracene-induced mammary tumors in rats. Breeder Sprague Dawley rats were fed AIN-93G diets in which soybean oil was replaced by corn oil and the protein source was either casein, whey, or soy-protein isolate. The soy diet contained isoflavones 430 mg/kg diet, specifically, genistein 276 mg/kg diet and daidzein 132 mg/kg diet. The study authors estimated that a 333 g rat eating 25 g diet/day would be exposed to genistein at 20.4 mg/kg bw/day. Rats from the same dietary groups were bred to produce F_1 offspring, and upon maturity, the F_1 offspring from the same dietary groups were bred to rats from different litters to produce F_2 offspring. **[The number of rats bred was not stated for any generation, and it is not known how many**

litters were represented in F_1 rats selected for breeding.] At weaning, the F_1 and F_2 offspring were fed the same diets as their dams and continued to receive the diets throughout their lifetimes. **[Based on a body weight figure presented in the study, it was estimated that a weanling (21-day-old) rat weighs 50 g. The EPA *(313)* assumption for food intake in a weanling female Sprague Dawley rat is 0.150 kg/kg bw/day. Therefore total genistein intake in weanling rats was estimated at ~830 mg/kg bw/day.]** At 50 days of age, ≥19 F_1 rats/group **[inconsistent values reported for number of whey-fed rats in various tables]** and ≥31 F_2 rats/group were gavaged with dimethylbenzanthracene 80 mg/kg bw and the development of palpable mammary tumors was assessed. Rats were killed when all rats from the casein diet had at least 1 palpable mammary tumor. Tumors were examined, fixed in buffered formalin, and sectioned. Statistical analyses included ANOVA, Kaplan-Meier analysis, Wilcoxon test, Fisher exact test, and Kruskal-Wallis test.

Beginning at 8 weeks of age, body weight gain in both generations of casein-fed rats was slightly but significantly higher than body weight gain in the soy- or whey-fed rats. Vaginal opening was significantly accelerated by 1 day in the soy-fed compared to casein- or whey-fed rats (PND 37 versus PND 38). No differences were observed for relative organ weight, estrous cycle, successful breeding, sex ratio, or litter size **[data not shown]**. Results for mammary carcinogenesis are outlined in **Table 150**. Latency, in terms of the number of post-treatment days for 50% of rats to develop at least 1 tumor, was significantly increased in both generations of the soy- and whey-fed rats compared to casein-fed rats. Time course for tumor development was accelerated in the casein-fed rats, as noted by a significantly lower percentage of soy- and whey-fed rats with tumors at the time when 100%

Table 150. Effect of Diet on Dimethylbenzanthracene-Induced Mammary Tumors in Female Rats (Hakkak et al., 2000)

Parameter	*Generation*	*Diet*		
		Casein	*Soy*	*Whey*
Days after dimethylbenzanthracene:				
To first tumor	F_1	28	42	28
	F_2	36	36	43
To 50% of rats with ≥1 tumor	F_1	52 a	65 b	83 b
	F_2	54 a	68 b	89 c
Rats with at least 1 mammary tumor at end of study, %	F_1	100 a	84 a,b	62 b
	F_2	100 a	77 b	54 b
Rats with at least 1 adenocarcinoma, %	F_1	95	90	86
	F_2	85	79	63
Rats with at least 1 mammary tumor with intraductal proliferation, %	F_1	5	5	5
	F_2	13	21	32
Tumors/rat, median (range)	F_1	5 (2–12) a	3 (1–12) a,b	2 (1–22) b,c
	F_2	3 (1–7) a	3 (1–10) a	2 (1–7) b
Median tumor volume, cm3	F_1	1.9	1.5	0.6
	F_2	3.0	1.8	0.8

a,b,c Within each row, numbers with different superscripts are statistically different from one another.
From Hakkak et al., 2000 *(713)*.

of casein-fed rats had developed a tumor. Tumor multiplicity (numbers of tumors/tumor-bearing rat) was significantly lower in both generations of whey-fed rats compared to casein-fed rats. There were no other significant differences in tumor parameters. The study authors concluded that soy-rich diets reduce dimethylbenzanthracene-mammary tumor incidence by about 20% in rats and that whey appears to be twice as effective as soy products in reducing tumor incidence and multiplicity.

Strengths/Weaknesses: A strength of the study is that rats were fed AIN-93 diets with casein, soy, or whey as the sole protein source for 2 generations. Dietary isoflavone, genistein, and daidzein concentrations were analytically determined. Animals were exposed through diet, a relevant route of exposure. Mammary tissue was examined by a pathologist who was blind to treatment group. In the statistical analyses, day of measurement was included to account for multiple measures per animal and interactions between diet and day were examined. Offspring were exposed to a soy-containing diet across critical windows of development. Dose levels were reportedly similar to those ingested by a 4-month-old infant consuming soy-based infant formula. A weakness is inclusion of only one dietary level of soy, which does not allow for evaluation of dose-response relationships. There were no analytical data presented for the stability of genistein in the diets, and no information as to how often diets were replenished. The authors did not state how many animals were bred in the parental or F_1 generation or how many litters were produced. It is not known whether litter effect was controlled (i.e., selection of one male and one female/litter for mating of the next generation and use of litter-based analyses, such that animals from the same litter represented an n of 1). The soy effect on lowering tumor incidence was only statistically identified in 1 of the 2 exposed generations described in this paper. Details were not provided for the assignment of animals into treatment groups (i.e., approximately equal mean body weights and variances across groups). It would have been useful if the authors had reported body weight at the time of puberty onset because a 1-day acceleration in vaginal opening in the soy-fed females likely occurred in animals at lower body weights than in casein controls. Furthermore, the mean age at vaginal opening in the female Sprague Dawley rats (38 days in casein-fed controls) is later than the value reported in many other publications (e.g., 33.4 days, range 31.6–35.1 days *(714)*). DMBA dose is a little high and may not readily detect increased tumorigenesis. However, this is mostly offset by the measure of time to 50% tumor incidence as an endpoint.

Utility (Adequacy) for CERHR Evaluation Process: This report is of limited utility in the CERHR evaluation process owing to the multigeneration exposure to genistein treatment and the comparison with other dietary proteins such as whey. As previously mentioned the use of DMBA to induce mammary tumors is of limited relevance to human breast cancer.

Mehta et al., 2006 ***(715)***, funded by Health Canada Genomics Initiative, examined the effects of dietary isoflavones on methylnitrosourea-initiated mammary gland cancer in F_1 female rats from parents who had undergone lifetime exposure to variable levels of NOVASOY^{TM} (NS), a commercial preparation containing a total isoflavone concentration of 24% (12% genistein, 9% daidzein, and 3% glycitein). Diet 1 was casein-based, AIN-93G semi purified basal diet containing AIN-93G Vitamin Mix and AIN-93G Mineral Mix; Diet 2 was soy-based basal diet containing alcohol-washed, isoflavone-extracted, soy protein, AIN-93G Vitamin Mix and AIN-93G Mineral Mix; Diet 3 was the soy-based complete basal Diet 2 supplemented with 40 mg/kg diet NS; Diet 4 was the soy-based complete basal Diet 2 supplemented with 1000 mg/kg diet NS. Female rats were culled at weaning from a multi-generational study in which the F_0 parents (Sprague-Dawley from CRL, Quebec) had

undergone lifetime exposure to Diets 1-4. At weaning (28 days), the rats were fed their parents' diet (Diets 1-4) and remained on the diet throughout the study. At 45 days of age, rats in each of the Diet Groups were assigned to one of two subgroups: Subgroup A received a single ip injection of vehicle (0.05% acetic acid in AIN-93G) normal saline); subgroup B received a single ip injection of methylnitrosourea (MNU) in vehicle at a dose of 40 mg/kg body weight. Thus there were eight treatment groups: 1A (n=11), 1B (n=14), 2A (n=12), 2B (n=14), 3A (n=19), 3B (n=20), 4A (n=20), and 4B (n=20); treatment group 1A received Diet 1 and was injected with vehicle on day 45; treatment group 1B received Diet 1 and was injected with MNU on day 45; treatment group 2A received Diet 2 and was injected with vehicle on day 45; treatment group 2B received Diet 2 and was injected with MNU on day 45, etc. Body weights and feed consumption were recorded weekly. Beginning week three after MNU treatment, the number and size of the mammary gland masses was recorded after palpation weekly. On the 168th day after MNU treatment, the rats were killed and necropsied. Mammary glands were retained in NBF for whole mount analysis and histopathology. Sections of all palpable mammary masses were examined as well as all #8 mammary glands without masses. Food consumption and body weight data were analyzed using 2-way Analysis of Variance (2-way ANOVA) for determinations of effects of diet and time, followed by pairwise multigroup comparisons by the Holm-Sidak method. Tumor incidence and average tumor size at necropsy from 11-20 animals per treatment group were analyzed by Kruskal-Wallis One-Way Analysis of Variance on Ranks (1-way ANOVA), with pair-wise comparisons conducted by Dunn's method. Time to first tumor appearance was analyzed using the Kaplan-Meier log-rank survival analysis, followed by pairwise multigroup comparisons using the Holm-Sidak method.

There was a statistically significant difference in body weight gain between treatment groups. For all diets, the saline-treated rats had a higher body weight gain compared to their respective MNU-treated rats; the casein control diet groups (1A and 1B) gained less body weight compared to their respective soy-based control diet groups (2A and 2B). Rats in the highest NS dose groups (4A and 4B) gained less body weight than their respective counterparts at the lower doses of NS (Groups 2A and 2B and Groups 3A and 3B); however the body weight gains in rats fed the highest dose of NS (4A and 4B) were similar to those fed the casein-based control diet (1A and 1B). Similar affects were seen on daily food consumption. The first tumors appeared in rats receiving NS at both dietary levels on day 77 after MNU injection. In Group 3B, the average tumor growth remained higher than that in Group 4B until necropsy. Tumor appearance in MNU-exposed rats in the NS-free diet groups was delayed until Day 98 in the casein-based diet group (Group 1B) and until day 112 in the soy-based diet group (Group 2B). These delays were not statistically significant. At the end of the 168-day study, the tumor incidence (number of rats with tumors) was higher for all MNU-treated groups compared to their respective vehicle-injected controls; these differences were statistically significant. The average number of tumors/rat was statistically significantly higher in rats exposed to MNU compared to their respective vehicle-injected controls. Tumor multiplicity was statistically significantly lower for the rats in the soy-based diet group (Group 2B) compared to the casein-based diet (Group 1B). There was a trend toward lower tumor multiplicity in rats fed a higher dose of NS (Group 4B) compared to the middle-dose diet (Group 3B), this trend did not achieve statistical significance. Overall a higher rate of tumor growth during the study, and larger tumors at the end of the study was seen in the Group 3B rats. There was no effect of basal diet or NS dose on tumor size. The mammary glands from Groups 1A, 2A, 3A and 4A exhibited pathology within the normal limits. In the rats injected with MNU, the data suggest an effect of the basal diet and NS dose on tumor type and histogenesis (**Table 151**).

Table 151. Effects of Diet on Mammary Gland Tumor Type after MNU Injection (Mehta et al., 2006)

Treatment Group	Tumor Type			
	Noninvasive Carcinoma, Ductal Papillary	*Noninvasive Carcinoma, Ductal Cribriform*	*Fibroadenoma, Benign*	*Tubular Adenocarcinoma*
1B (non-soy diet)	[a] 73 (4/6) [b]	27 (2/6)	0	0
2B (basal soy diet)	29 (2/6)	71 (4/6)	0	0
3B (basal soy diet + 40 mg NS/kg diet)	21 (2/10)	79 (7/10)	0	0
4B (basal soy diet + 1000 mg NS/kg diet)	0	80 (6/8)	10 (1/8)	10 (1/8)

[a] % of total lesions examined.
[b] Number of animals with the tumor/Number of tumor-bearing animals.
From Mehta et al., 2006 *(715)*.

The carcinomas in this study were locally expansive encapsulated lesions that affected the normal mammary gland architecture, but there was no evidence for invasion or metastases in any of the treatment groups.

Authors' conclusion: An evaluation of a dose-response relationship pointed towards a biphasic effect, with a trend showing lower tumorigenesis at 1000 mg/kg diet NS compared to 40 mg/kg diet NS, thus corroborating the previously suggested dual properties of isoflavones as estrogens agonists, antagonists, and/or selective estrogen/progesterone receptor modulators.

Strengths/Weaknesses: A strength of the study design is the use of more than one dose group for genistein and dietary route of exposure. Body weight and feed consumption measured weekly is another strength allowing the authors to adjust their dose levels to account for weight gain in the study animals. Focus solely on tumor formation and absence of reproductive measures such as time to vaginal opening, estrous cycle characteristics is a weakness of this study. Relevant exposures through dams and then orally once weaned. AIN-based diets are appropriate but precise concentrations of isoflavones (based on NovaSoy extracts) in these diets is not immediately clear. Body weights and feed consumption recorded. However, rats were maintained on their parents diet throughout the study (apparently through until the 168th day following NMU treatment) and at 45 days of age received either saline or NMU. Dosage of NMU is close to optimum and may be difficult to detect increases in tumorigenicity. Overall interpretations may be biased towards detecting only protective (or apparently no-effect) outcomes. Use of MNU to induce tumors, although an acceptable model, has limited relevance to human breast cancer. There are no useful developmental endpoints measured other than gross body weight.

Utility (Adequacy) for CERHR Evaluation Process: This study is of no utility for further evaluation because of the lack of endpoints useful to assess effects of the diets on development, and the exposure throughout the study period (effectively "lifetime" exposure)

Rowlands et al., 2002 ***(716)***, funded by the USDA, examined the effects of a soy-protein isolate diet on mammary development in rats. Beginning on GD 4 **[day of plug not specified]**, 10 pregnant Sprague

Dawley rats/group were randomly assigned to receive 1 of 3 AIN-93G diets containing soy protein isolate, whey, or casein as the protein source. The diets were equivalent in calories, protein, and amino acid content. **[A statement was made that the animals were exposed throughout their lifetimes, and it was therefore assumed that the animals were fed the assigned diets through the remainder of gestation and the entire lactation period.]** Offspring were weaned to the same diets as their dams. On PND 21, 33, and 50, one female/litter/group (n=10/time period) was killed for examination of mammary gland morphology. The same diets and exposure protocol were used in a second experiment in which offspring were selected from 5–7 litters and were weaned to the same diets as their mothers. Around PND 48–51, 1 female/litter/group (n=5–7/group) was gavaged with sesame oil and killed 24 hours later in metestrus. Mammary glands were removed for examination of cell proliferation and expression of ERα, ERβ, and progesterone receptor. The litter was considered the statistical unit in analyses that included ANOVA and Student-Newman-Keuls multiple comparison tests.

Mammary gland area in rats of the soy diet group was 36–38% larger than in rats of the casein and whey protein groups on PND 50. This effect was not observed on PND 21 or 33. A 4-fold (75%) decrease in terminal end bud density was identified in rats of the soy and whey protein diet groups compared to the casein diet group on PND 50, but the effect did not attain statistical significance. No significant differences in alveolar or lobuloalveoli bud density were detected. Terminal end bud cells expressing progesterone receptor were increased by 34% in the soy protein compared to the casein diet group at 48–51 days of age, but this effect was not seen in lobular cells. No significant effects of the soy protein isolate diet on proliferation or expression of ERα or ERβ in terminal end buds or lobular cells were detected. The study authors concluded that the soy protein isolate diet stimulated mammary gland differentiation.

Strengths/Weaknesses: A strength was that rats were fed AIN-93 diets with casein, soy protein isolate or whey protein hydrolysate as the sole protein source. The authors controlled for litter effect, using either one female pup/litter for an endpoint and/or using the litter as the unit of analysis. The methods for scoring mammary whole mounts and immunohistochemistry samples were clearly described. The authors controlled for stage of estrous cycle when proliferation index was determined in mammary glands. A negative control (omitting the primary antibody) was included in immunohistochemistry experiments to verify receptor populations. Animals were exposed in the diet, a relevant route of exposure. The statistical analyses were appropriate. Offspring were exposed to a soy-containing diet across several critical windows of development and evaluated at ~50 days of age. A weakness is that the study included only one dietary level of soy, which does not allow for evaluation of dose-response relationships. Blood levels of phytoestrogens (e.g., genistein) were not measured. The authors did not specify whether the sperm-positive day was designated as GD 0 or 1. There were no analytical data presented for the stability of genistein in the diets and no information as to how often diets were replenished. Dietary levels of genistein and daidzein were not specified (perhaps these levels were the same as Hakkak *et al.*, 2000 *(713)*). No data on body weight were presented. Sample sizes were likely insufficient for endpoints such as mean number of terminal end buds/mm^2, given that a 75% decrease was not statistically different. Details were not provided for the assignment of animals into treatment groups (i.e., approximately equal mean body weights and variances across groups).

Utility (Adequacy) for CERHR Evaluation Process: This report has no utility for the evaluation.

Simmen et al., 2005 ***(717)***, supported by USDA, examined the effects of soy diet on chemically induced mammary tumorigenesis. On GD 4 (day of plug not stated), Sprague Dawley rats were randomly assigned to groups fed AIN-93G diets containing either casein or soy-protein isolate as the sole protein source. **[Based on numbers of pups reported later in the study, it appears that 10 dams/group received each diet.]** Dams were allowed to litter, and pups from the same treatment groups were pooled. Five pups/sex/group were randomly placed with dams from the same treatment groups. The 50 female pups/group were weaned on PND 21 (day of birth not defined) and fed the same diets as their dams. On PND 50, female offspring were iv injected with 50 mg/kg bw *N*-methyl-*N*-nitrosourea. Rats were weighed weekly, palpated twice/week for mammary tumors, and killed 115 days following *N*-methyl-*N*-nitrosourea treatment. Mammary tissues were subjected to immunohistochemical analyses to determine proliferative cell nuclear antigen and apoptosis. RNA was isolated from mammary tissues and reverse transcription-polymerase chain reaction was used to determine expression for a number of genes involved in cell proliferation, cell differentiation, and apoptosis. Serum estrogen **[not otherwise identified]** and progesterone levels were measured. MCF-7 breast cancer cells were incubated in sera obtained from each diet group in order to measure apoptosis. Data were analyzed by ANOVA or Student *t* test.

Compared to rats fed the casein diet, rats fed the soy-protein isolate diet had increased mean ± SEM tumor latency (93.0 ± 4.3 vs. 78.0 ± 3.9 **[unit assumed to be day]**; $P<0.05$) and decreased incidence of rats with at least 1 tumor (57.4 vs. 83.3%; $P<0.01$). Rats fed the soy-protein isolate diet had a lower incidence of ductal carcinoma *in situ* (42 vs. 64% $P=0.08$) but a higher incidence of infiltrating ductal carcinoma (55 vs. 33%; $P=0.086$). Diet had no detected effect on cell proliferation or apoptosis. Progesterone receptor and *HER-2/neu* (involved in cell proliferation) gene expression were increased in rats fed the soy-protein isolate diet. Serum progesterone levels were ~7 times lower in rats fed the soy-protein isolate diet, but there was no detected effect on serum estrogen level. Apoptotic cells in an MCF-7 culture were modestly but significantly increased following incubation with sera from rats fed the soy-protein isolate compared to the casein diet **[data not shown]**. The study authors concluded that lifetime exposure to soy-protein isolate protected rats from *N*-methyl-*N*-nitrosourea-induced mammary carcinogenesis and altered signaling pathways, including progesterone receptor and *HER-2/neu*.

Strengths/Weaknesses: Dietary route of administration is relevant. Animals were randomly assigned to each group; may have created "unequal" groups (e.g., body weight distribution); not clear that litter was the unit for analysis. It is a strength that the amino acid content of the diets was equalized although diets were not analyzed for stability, concentration, or homogeneity. A potential weakness is that only 1 dose level of soy was used and only female rats were evaluated. However, this was a study of mammary carcinogenesis and the use of males would likely be uninformative**.** Soy was given over a lifetime, making the study difficult to interpret for the current evaluation even thought that may model consumptions in Asian populations. Use of the NMU to induce tumors raises questions concerning the relevance of the data for human breast cancer. Reproductive functional data such as time to vaginal opening and estrous cycle is noticeably lacking in this study. Dosing of MNU may not easily detect increased tumorigenesis; thus, data and overall interpretations may be biased towards detecting only protective (or apparently no-effect) outcomes. Cell culture studies are poorly designed and difficult to interpret, e.g., high concentrations of estradiol in fetal serum will compete with genistein for ER effects. The study did not include endpoints that would have been useful to assess effects of the diets on development. While tumorigenesis can be a meaningful endpoint in developmental studies, e.g., if

the consequences of the developmental changes alter lifetime cancer risk; the absence of informative measurements on the normal glands and the "lifetime" (115 days post NMU) exposure are not useful to address the goals of the process.

Utility (Adequacy) for CERHR Evaluation Process: This study has limited utility for the evaluation process due to the lifetime exposure to soy and the focus on tumor detection as a primary endpoint. The assessment of progesterone receptor and *HER-2/neu* expression is potentially useful information.

Su et al., 2009 ***(718)***, supported by the USDA, conducted genome-wide profiling of mammary tissues of weanling rats exposed to soy protein isolate (SPI) or control casein via maternal diet to evaluate the contribution of early exposure on mammary gene expression. *In vivo*: Semipurified diets were prepared according to the AIN-93G with corn oil substituted for soybean oil and containing either casein (control diet) or SPI as the sole protein source. SPI contained 3.21 g/kg total isoflavones, including 1.87 g/kg genistein-containing compounds and 1.22 g/kg daidzein-containing compounds. Pregnant Sprague Dawley rats (Charles River Laboratories) were assigned to one of the two test diets on gestation day 4 and remained on these diets through the duration of the experiment **[daily exposure levels not stated)] [number and age of dams not stated] [bedding not described] [basis of dose selection not described]**. On PND 21, five female pups per group were killed and the inguinal mammary glands (#4) were removed. The right gland was homogenized for RNA extraction, portions of the left gland were fixed for paraffin embedding and stained with H&E for determination of adipocyte size; and the remaining gland was frozen in liquid nitrogen. Three female weanlings per group continued on the same diet as their mothers until PND 50 when they were killed, the abdominal (retroperitoneal) fat pad was removed and weighed, and the inguinal mammary glands (#4) were removed. The right mammary gland was prepared for whole mount analysis and the number of TEB (terminal end buds) was evaluated; the left gland was processed as for the PND 21 pups. Total RNA was extracted from the mammary tissue of the PND 21 rats; RNA isolation, microarray, gene expression data analysis and hierarchical clustering were performed. Quantitative RT-PCR and Western blot analysis were performed. *In vitro*: Mouse mammary epithelial HC11 cells were incubated in conditioned medium (CM) harvested from genistein (0.5 μmol/L) or vehicle-treated differentiated 3T3-L1 adipocytes, in the presence of prolactin, and were evaluated for *Csn2* gene expression status. Both Experiments: Differences between diet groups were assessed by Student's *t* test, one-way ANOVA, and two-way ANOVA followed by Tukey's test.

In vivo: Female rats exposed to dietary SPI (soy protein isolate) had lower body weights at PND 21, 33, and 50, and lower abdominal fat weight at PND 50 compared to the control (casein) rats. At PND 50, rats of the SPI group tended to have fewer TEB and higher mammary β-casein (*Cas2*) and *Wap* transcript levels relative to control rats of the same age. The gene expression profiles of the whole mammary glands of developing rats were examined–of the 14,280 unique genes analyzed, 57 transcripts were differentially regulated by dietary SPI relative to the casein controls. Among these, 18 were induced and 39 were repressed. Of the SPI-regulated metabolism-related genes, 12 were associated with lipid metabolic pathways. Genes related to adipocyte lipogenesis were down-regulated by dietary SPI; also down-regulated were genes that regulate cholesterol biosynthesis and fatty acid transport. Dietary exposure to SPI resulted in lower mean fat cell size compared to casein control rats. The differential expression of *Hsd11b, Scd1, Me1, Dhcr7, Fasn,* and *Acly* with diet was confirmed by RT-PCR. At a dose (0.5 μmol/L) comparable to that found in sera of the PND 50 rats exposed to lifetime dietary SPI, genistein reduced the transcript levels of *Fasn* and *Scd1* genes and augmented the expression of the

Hsd11b1 gene in differentiated 3T3-L1 cells. The changes in *Hsd11b1* and *Fasn* gene expression were confirmed for the corresponding proteins by Western blot. The transcript levels of nuclear receptor PPARγ2 and of the adipokine leptin were unaffected by genistein. *In vitro*: The protein levels of CM harvested from control (vehicle) and genistein-treated adipocytes did not differ. CM from genistein-treated adipocytes had higher levels of immunoreactivity adiponectin than CM from control-treated adipocytes at 24 hours, which were further increased at 48 hours. Expression of *Csn2* was not detected in HC11 cells treated with either CM in the absence of Prolactin. With added Prolactin (1 or 5 mg/L), however, epithelial cells treated with genistein-CM had higher *Csn2* transcript levels than those treated with control CM. HC11 cells, when pretreated with the glucocorticoid antagonist, RU486 (1 μmol/L), prior to the addition of genistein-CM had a loss of *Csn2* gene expression relative to genistein-CM alone in the presence of Prolactin. RU486 in the absence of Prolactin did not affect *Csn2* expression.

Authors' conclusion: The findings suggest that soy-associated components, by targeting mammary adipocytes, alter paracrine signaling to enhance mammary epithelial differentiation, with important implications for the prevention of breast cancer associated obesity and obesity-related diseases.

Strengths/Weaknesses: This is a study that looked at the effects of early soy exposure (by maternal diet) on mammary gland gene expression. The study addresses a relevant subject from a mechanistic perspective. The use of the AIN-based diet is sound but it is not clear what other isoflavones are present in the supplement beyond those specifically measured. Thus, it may be difficult to attribute any specific gene expression changes to genistein or any other component of the soy protein isolate supplement. The gene expression array studies seem to have been competently done from a technical perspective. However, the approaches to data analysis are simplistic and the data are somewhat over-interpreted and speculative. While differential expression of a few genes was confirmed, no studies were done to establish the functional relevance of these changes; independent validation of the differential expression of genes was rather limited. It is to be expected that many genes will change but separating noise from truly regulated genes, and then establishing their functional relevance, requires a more detailed and sophisticated approach that that evident in this study. The author's interpretations are not well substantiated by the data, and while they are intuitively logical there is no experimental evidence to establish that they are correct. It is understood that such studies are largely hypothesis generating, but a more effective analysis of the data might have generated more confidence in these speculations.

Utility (Adequacy) for CERHR Evaluation Process: This study has no utility for the primary goals of this project. It is possible that the data could be used to obtain new hypotheses regarding what molecular pathways are modified by an early life exposure to isoflavones.

Thomsen et al., 2006 ***(719)***, supported by the Commission of the European Communities: FAIR program, examined the effect of 17β-estradiol (E2) and soy isoflavones exposure on morphogenesis and global gene expression in the murine mammary gland. FVB mice were obtained from Taconic M&B (Berlin Germany), the animals were fed a semisynthetic diet based on casein (18%) and carbohydrates (68%). Two types of diet were prepared: a control diet and the control diet to which was added 0.06% wt/wt (270 mg/kg diet) of Prevastein (46.1 % isoflavones, of which 66.5% was genistein, 32.3% daidzein, and 1.2% glycitein). 144 female pups from 35 dams, were included on study. The pregnant mice were kept on control diet until delivery (PND 0), after which they either continued on the control diet (n=25) or the control diet supplemented by Prevastein (n=10) **[bedding**

not described]. After weaning on PND 21, the litters of the dams that had received control diet were divided into three treatment groups: control diet, control diet plus Prevastein, or control diet plus E2 (**[purity not stated]** 2.5 mg/kg body weight) administered orally as a droplet (~5 µl of 5 mg/ml) on PNDs 10, 12, 14, 16, 18 and 20. The litters from dams fed the Prevastein-containing diet were switched to control diet. Thus there were 4 treatment groups: (I) control diet (n=42 F_1 females), (II) 270 mg isoflavone/kg diet throughout lactation (PND 0-21, n=38), (III) 270 mg isoflavone/kg diet from weaning through the end of the study, n=38), and (IV) E2 during the lactation period (n=26). Body weights and feed consumption (after weaning) were recorded weekly. Mammary glands and blood were collected on PND 28, 42-43, and 70-73. Animals were killed while in estrus (except PND 28). Concentrations of daidzein, genistein, and equol were quantified in 5 samples per group by time-resolved fluoroimmunoassay; estradiol concentrations were measured in 3 to 5 animals per group. Mammary whole mounts were prepared from the 4th abdominal mammary gland (on PND 28, n=8-12; on PND 42-43, n=10-16; on PND 70-73, n=10-14). The length, branching, morphogenesis, stage of differentiation, terminal end buds (TEBs), terminal ducts, and alveolar buds were analyzed. All data were tested for normal distribution by Shapiro-Wilks test; homogeneity of variance among the groups was evaluated by judgment of standardized residuals plot. Data on mammary gland morphology were analyzed by one-way analysis of variance followed by Student's *t* test for the means (unpaired and two-tailed) for pairwise comparison. Values of $P<0.05$ were considered statistically significant. RNA was isolated from the 3rd mammary gland (n=3-5). Expression ratios were log-transformed and the normalized data were analyzed by Statistical Analysis of Microassays (SAM) and hierarchical clustering. cDNA was prepared from 2 µg RNA via polymerase chain reaction (PCR); real-time PCR was performed (n=3-5 samples). Tissue sections for *in situ* hybridization (ISH) were prepared from the 4th abdominal mammary gland (n≥3). Immunohistochemistry (IHC) was investigated in tissue sections prepared from the 4th abdominal mammary gland (n≥3).

At PND 28, animals in Group II had higher isoflavone metabolite plasma levels than the animals on the control diets. The plasma levels of estradiol were comparable among all exposure regimens. The mammary trees from E2-treated animals were shorter, more branched, and contained more TEBs than glands from animals on the control diet; isoflavone -treated animals seemed only to differ from controls by more branching. Morphometric analysis of the mammary epithelium showed no difference in the relative length of the mammary tree (except for the shorter glands in the E2 group on PND 42-43). A significant increase in overall branching in the juvenile mammary gland (PND 28) was observed in all exposure groups (Groups II-IV). At PND 42-43, increased branching was only observed in glands from the E2-treated animals (Group IV). In the juvenile mammary gland, the number of TEBs was only significantly affected in the E2 treatment (Group IV); a similar effect was found after postweaning isoflavone treatment (Group III). At PNDs 42-43, a significant reduction in the number of TEBs was evident after postweaning isoflavone treatment and E2 treatment (Groups III and IV). There was no effect observed on the adult animals (PNDs 70-73). The level of plasma estradiol was comparable between the groups and among the samples chosen for the microassay. The three treatments yielded very similar gene expression profiles. No genes were found to differ in expression level when comparing the postweaning isoflavone exposure to the E2 exposure, and very few genes were differently expressed when comparing the lactational isoflavone exposure to the E2 exposure. Ten genes regulated in all three treatments were further studied by RT-PCR; for most of the analyzed genes, RT-PCR confirmed the differential expression. There was no difference in the intensity of the IHC and ISH staining, but TEBs from the control animals were smaller and less

developed compared to TEBs from E2- and isoflavone-treated animals. IHC with antibodies against AP-2γ showed expression in cap/transition and body cells of the TEBs; the staining of all cell types showed a mixed pattern with both positive and negative cells. There was no difference in staining intensity for Crk or for the expression of Clusterin (apolipoprotein). Real-time PCR indicated a downregulation of more than twofold for both isoflavones and E2 treatments during the prepubertal period. Staining of control mammary glands with an ERα antibody seemed qualitatively to be slightly more intense than the treated glands.

Authors' conclusion: The results suggest an estrogenic response of physiological doses of isoflavones on mammary gland development at both the morphological and molecular level, which resembled that induced by puberty.

Strengths/Weaknesses: Use of dietary route of exposure and relevant timing of exposures are strengths of this report. Addition of Prevastein to the control diet provides insight into the effect of a commercially available soy supplement. The semi-synthetic diet is not defined with sufficient detail to know if appropriately controlled for sources and levels of macro/micronutrients. Not immediately clear that this base diet is isoflavone free; if not then the study is seriously flawed. The utility of this study would be greatly enhanced if this could be definitively determined (the relatively low mean concentrations and modest variances for the genistein data in Table 1 suggest that this may be an isoflavone "low" diet). Diets were not analyzed for stability, concentration, or homogeneity; nor is it evident that the isoflavone concentrations reported were those in the diet–they appear to be those in the isoflavone product used to prepare the diet. Four diets were used but it is not clear that a control of E2 exposure from weaning through to the end of the study was included. Single exposures do not allow for dose response analysis. Pups were cross-fostered, although how they were randomized is unclear.

Mammary glands were examined at three time points. Assessment of molecular markers thought to be important in mammary gland development is a another strength of this report. Use of one gland (fourth mammary gland) to prepare whole mounts and the isolation of RNA from the third mammary gland adds mechanistic insight and takes full advantage of experimental material available for assessment. Analysis for circulating concentrations of daidzein, genistein, and equol by time-resolved fluoroimmunoassay is another strength of this report. Levels of the individual isoflavones is useful; the total isoflavone values probably have no utility, since each compound will have different affinities for different (and sometimes the same) molecular targets and likely each has a different pattern of biological activities (even if these have some overlap). Focus on mammary gland development and the lack of reproductive or developmental data is a limitation of the present report.

Utility (Adequacy) for CERHR Evaluation Process: This report has limited utility for the evaluation process owing to the focus on mammary gland tumor development and the lack of reproductive or developmental data.

3.4.3 Brain and Behavior Endpoints

Becker et al., 2005 ***(720)***, supported by the University of Evansville and NIH, evaluated effects on neonatal behavior of dam treatment with a dietary phytoestrogen supplement during pregnancy. Female Sprague Dawley rats were randomized to 1 of 3 diets from the beginning of the second week of pregnancy until weaning. Two of the diets were described as "normal" and consisted of commercial chows (Harlan

Teklad 8604, n=4 dams, and Purina 5001, n=8 dams). The third diet was a low-phytoestrogen chow (Harlan Teklad 2014, n=21 dams). Eleven of the 21 dams receiving the low-phytoestrogen diet were given 2 daily phytoestrogen supplement tablets, which together contained daidzein 34 mg, glycitein 20 mg, and genistein 8 mg. Complete consumption of the supplement tablets was assumed based on failure to find tablet remnants in the cages. Daily genistein intakes were estimated based on mean daily feed intake and supplement composition to be 19.45 µg in the rats on the low-phytoestrogen diet, 322.26 µg in the rats given the "normal" diets, and 1287.30 µg in the rats given the low-phytoestrogen diet plus the phytoestrogen supplements. **[Dam body weights were not given. Assuming a dam body weight of 250 g, genistein intakes would have been 0.08 mg/kg bw/day on the low phytoestrogen diet, 1.3 mg/kg bw/day on the "normal" diets, and 5.1 mg/kg bw/day on the phytoestrogen-supplemented diet.]** Dams were permitted to litter, and pup anogenital distance and body weight were measured on the day pups were found (PND 1). At 24–48 hours of age, litters were standardized to 5 males and 5 females, with fostering of pups within treatment groups if necessary to achieve standard litters. Litters were eliminated from consideration if pup counts fell below 8. Righting reflex was assessed in 1 male and 1 female pup from each litter on PND 3, 5, and 7. Ultrasonic vocalizations on separation from the dam were counted on PND 5, 10, and 15. Litters were weaned on PND 21–22. Between PND 70 and 100, males were anesthetized and cardiac puncture used to obtain blood samples for measurement of plasma corticosterone and testosterone. Statistical analysis was performed with Dunnet *t* test.

There were no detected treatment-related differences in length of gestation or number of male or female offspring. The groups that received the low-phytoestrogen diet had lower percentages of deliveries and of litters surviving to testing than did the groups that received the "normal" diets, attributed by the authors to the lower protein content of the low-phytoestrogen diet. Anogenital distance corrected for body weight was increased in males and females on the low-phytoestrogen diet without phytoestrogen supplementation compared to the "normal" diets. Pups in the "normal" diet groups gained more body weight during the lactation period than pups in the low-phytoestrogen group, with pups from the phytoestrogen-supplemented group intermediate in body weight between the other 2 treatment conditions. Righting reflex did not show significant treatment effects. Pups of both sexes from the low-phytoestrogen group emitted more ultrasonic vocalizations at most tested times, although not all of the apparent differences were statistically significant. When dams were given phytoestrogen supplements and the low-phytoestrogen diet, pup vocalizations were similar to those in the "normal" diet groups. There were no detected treatment-related changes in plasma corticosterone or testosterone.

The authors speculated that rats in the low-phytoestrogen group did not experience the anti-anxiety effects of dietary estrogens, resulting in increased ultrasonic vocalizations in pups after separation from the dam. The authors acknowledged that they could not separate estrogen exposure of the dam from exposure of the pups through milk or through direct consumption of supplement pills during the latter part of the lactation period.

Strengths/Weaknesses: A strength of the study is use of adequate numbers of animals. Two dose levels of genistein were tested, both in the relevant range, but mixed with other ingredients. Classic reproductive endpoints were examined (anogenital distance, body weight, plasma steroids) as well as 2 novel endpoints representative of behavior: the measure of ultrasonic vocalization (anxiogenic reflex) and righting reflex (motor development). A weakness is that actual exposure levels are uncertain due administration of genistein through feed and tablets. Another weakness is that the diets differed in %

protein. The low phytoestrogen diet which had the greatest effects on AGD and ultrasonic calling was the lowest protein (14.5% as compared to 23-24% for the two normal diets).

Utility (Adequacy) for CERHR Evaluation Process: This study is of no utility due to the uncertainty about doses. Speculation is required in interpretation of this study, but the results suggest caution regarding consumption of phytoestrogen tablets during pregnancy.

Golub et al., 2005 ***(721)***, supported by the Violence Research Foundation, evaluated neurobehavioral effects of soy formula in rhesus monkeys. The authors explored the hypothesis that manganese content of formulas would lead to neurobehavioral differences, noting that soy formula has a greater manganese content than does cow-milk formula. Infant monkeys were transferred on the day of birth to a primate nursery and assigned to 1 of 3 feeding groups. **[Randomization was not specified, but no feeding group differences in dam characteristics or infant birth weights were detected.]** A cow-milk formula group was fed Similac® with Iron, which contains manganese 50 µg/L. A soy-formula group was fed Baby Basics (a private-label soy formula available at Albertson's), which contains manganese 300 µg/L. A third group was given the soy formula with manganese added to give a concentration of 1000 µg/L. Infants were hand-fed for 2 weeks and then progressed to a sipper bottle. At 4 months of age, the monkeys were transitioned to a primate lab diet with supplemental grains, vegetables, and fruit. Behavioral observations and formal neurobehavioral testing occurred over the first 18 months of life. Cerebrospinal fluid was sampled under anesthesia at 4, 10, and 12 months of age and analyzed for homovanillic acid and 5-hydroxyindoleacetic acid. Statistical analyses were performed using ANOVA with post hoc Fisher test. When there was an apparent effect of soy formula, regression analysis was performed across the 3 formula groups to evaluate the manganese dose-relatedness of the effect. Manganese dose was calculated based on formula intake during the first 2 weeks of life.

No feeding group effects on body weight or length were detected during the first 4 months of life, when the formulas were being given. Between 6 and 9 months of age, the groups that received soy formula lagged "slightly" in height and weight **[data not shown; individuals not weighed or measured after 9 months]**. No significant feeding group effects on cerebrospinal fluid levels of homovanillic acid or 5-hydroxyindoleacetic acid were detected. Soy-fed infants initiated more behaviors than did cow milk-fed infants. Wake periods were shorter and sleep periods longer in soy formula-fed compared to cow milk formula-fed monkeys at 8 months of age but not at 4 months of age. Initiation and participation in play activity was decreased among soy formula-fed monkeys compared to cow milk formula-fed monkeys. This effect showed a significant correlation with manganese intake in the first 2 weeks of life. Behavioral response to apomorphine injection was also correlated with manganese intake. Temperament ratings and frequency of stereotypy were not affected by feeding group status. Infants in the soy formula-fed group were described as participating less readily in the structured testing, and the authors suggested that testing at an older age would be useful.

The authors concluded that integration of the behavioral findings was difficult but that increased behavioral changes, altered diurnal rhythms, and reduced play behavior may indicate altered regulatory control.

Strengths/Weaknesses: A strength of the study is that the dosing scenario is directly relevant to human exposures. Body weights and formula intakes were reported to allow estimation of doses administered.

Endpoints were evaluated from birth to 18 months of age to determine longer-term effects of neonatal exposure. The experimental model was the rhesus monkey, which is similar to humans in brain maturation at the time of birth. Infant monkeys were reared under standardized conditions (not specified). In order to assess a variety of behaviors, the authors selected a pre-determined number of sessions during which data for different endpoints were collected. Not all infant monkeys could generate useful data during the limited test sessions, so participation criteria were established for each test to identify data for exclusion. Observer reliability for scoring motor and postural behavior was established prior to the experiments. For social observations, infants were paired with a different infant for each session (one with whom they had not interacted socially) and monitored by a familiar observer. To alleviate inter-observer variability within a test, one observer collected all social dyad observations and another recorded all stereotypy observations. For the cognitive testing, performance criteria were established. Once these criteria were exceeded, a switch or reversal of the reward was introduced. A weakness of this study was that it used a single dose level of soy formula, which does not allow for an evaluation of dose-response relationships for soy formula (although the dose-response relationship for manganese could be evaluated). There was no information provided as to how infants were randomized into the various treatment groups (although pregnancy housing conditions were controlled across treatment groups). The authors stated that body weight and lengths were affected in the soy-fed animals between 6 and 9 months of age, but these data were not shown. Apparently, individual weights were not collected after 9 months of age (monkeys were weighed as a group). Cow-milk and soy formula differ in many other factors aside from soy, including nutritional components related to protein, carbohydrate, and fat. Sample sizes were relatively small for some neurobehavioral endpoints (n=8/group, although this sample size is good for a primate study). At 4 months of age, the primates were transitioned to commercial nonhuman primate diet with enrichment foods. A detailed analysis of the formula and diet contents was not reported, so actual exposures to phytoestrogens are not known. It is not known whether these dietary components contributed additional phytoestrogen exposures. Because social observations were discontinued at different times, the largest number of sessions completed by all monkeys was used for analysis; thus, some data were discarded. Cerebrospinal fluid metabolites and social interaction endpoints were not highly correlated. Infants performed the delayed non-match to sample task at chance level, indicating that the young monkeys were unable to learn this task.

Utility (Adequacy) for CERHR Evaluation Process: This report is of no utility in the CERHR evaluation process.

Lephart et al., 2001 ***(232)***, supported by the National Science Foundation and Brigham Young University, examined the effects of lifetime phytoestrogen exposure on anteroventral periventricular nucleus structure, aromatase level, and prostate weight in rats. Long Evans rat offspring used in this study were born to dams (at least 8/group) fed a diet containing phytoestrogen glycosides 600 µg/g diet (phyto-600) or a phytoestrogen-free diet throughout the gestation and lactation period. **[Distribution of offspring by litter was not discussed.]** According to a reference provided in this study *(247)*, protein sources were soy for the phyto-600 diet and fish meal for the phytoestrogen-free diet. Diets were reported to have equivalent amounts of protein, amino acids, carbohydrates, fats, vitamins, and minerals. F_0 breeders and F_1 offspring were examined in a series of experiments. Data were analyzed by ANOVA followed by Bonferroni post hoc comparison.

Experiment 1 examined effects in male and female breeders that had been exposed only during adulthood. Levels of phytoestrogens in brains of adult male breeders are reported in **Section 2.1.2**. In experiment 2, offspring were weaned at 30 days of age to the same diets as their dams. Body and prostate weights were measured at 75 days of age in 7–8 rats/group. Compared to rats fed the phytoestrogen-free diets, rats in the phyto-600 group had significantly lower body weight **[17% lower]**, absolute prostate weight **[31% lower]**, and relative (to body weight) prostate weight **[17% lower]**. Brain aromatase activity in the anteroventral periventricular nucleus area was measured at 110 days of age (n=6/sex/group) and reported to be unaffected by diet.

In experiment 3, half of the offspring fed the phyto-600 diets continued to receive that diet and the other half were given the phytoestrogen-free diet for 40 days, beginning at 80 days of age. Both groups were killed at 120 days of age for measurement of the volume of the anteroventral periventricular nucleus (n=5–7/sex/group). Males and females fed phyto-600 diets throughout their lifetimes displayed the typical pattern of ~2-fold higher anteroventral periventricular nucleus volume in females compared to males. Males switched to the phytoestrogen-free diet displayed a significant increase (nearly 2-fold) in anteroventral periventricular nucleus volume compared to males who remained on the phyto-600 diet. No effect of the change in diet on anteroventral periventricular nucleus volume in females was detected.

The study authors concluded that phytoestrogens decrease body and prostate weight and affect anteroventral periventricular nucleus volumes in adult males but have no effect on brain aromatase levels.

Strengths/Weaknesses: A strength of the study was that brain concentrations of daidzein, genistein, and equol were measured in the medial basal hypothalamus and preoptic area, thus verifying that these phytoestrogens reached the hypothalamus. Animals were exposed to phytoestrogens in the diet, which is a relevant route of exposure. Diets were analyzed by HPLC to determine concentrations of phytoestrogens, genistein, and daidzein (although this determination appears to have been made in previous studies). Validation of the time-resolved fluoroimmunoassay method used to measure brain concentrations of phytoestrogens has been published previously. Ovarian tissues were used as a positive control in the aromatase assay. The anteroventral periventricular nucleus sections were traced by two researchers blind to treatment group, and these traced areas were averaged. A weakness is that this study included only one dietary level of soy, which does not allow for evaluation of dose-response relationships. The stability of phytoestrogen-containing diets was not reported. The authors do not provide any litter parameters (e.g., fertility, litter sizes, sex ratio, pup body weights). They also did not provide any body weights at the time of randomization, during gestation and lactation, or for pups at weaning. It is difficult to discern whether the 17% decrease in body weight in the 75-day-old males maintained on the phytoestrogen-containing diet may have contributed to the alterations in prostate weight. Chapin *et al.*, 1993 *(722)* reported a significant (19%) decrease in absolute prostate weights in feed-restricted male rats with a 12% change in body weight. Thus, prostate weight can be influenced by body weight. It is not clear whether the authors controlled for litter effects (i.e., consideration of the litter as the unit of analysis for animals with lifelong phytoestrogen exposures; fetuses within the same litter exposed to phytoestrogen-containing or phytoestrogen-free diet *in utero* are not independent samples). Equol antiserum showed some cross-reactivity with dihydrogenistein and dihydrodaidzein. There was no phytoestrogen-free control included in the experiment measuring anteroventral periventricular nucleus volume. The experiment compared rats maintained on a

phytoestrogen-containing diet with those switched to a phytoestrogen-free diet at 80 days of age. It would have been useful to determine whether the early, *in utero*/prenatal/prepubertal exposures to phytoestrogens contributed to long-term differences in anteroventral periventricular nucleus area. Protein sources for these diets (per Lund *et al.*, 2001 *(247)*) were soy for the phyto-600 diet and fish meal for the phytoestrogen-free diet. The protein sources likely contributed other differences aside from phytoestrogen levels. Odum *et al.*, 2001 *(622)* reported that different rodent diets with different amounts of phytoestrogens had centrally mediated effects on rodent sexual development due to nutritional differences between the diets rather than ER-mediated effects. The authors do not discuss how these data relate to human hypothalamic development.

Utility (Adequacy) for CERHR Evaluation Process: This report has limited utility in the CERHR evaluation process owing to the single dose group used and the lack of fertility data in the report. The result of an increased AVPV in males ingesting soy diets is in agreement with other studies and suggests an anti-apoptotic role for GEN.

Lund and Lephart, 2001 *(723)*, supported by the National Science Foundation and Brigham Young University, examined the anxiolytic effects of a phytoestrogen-containing diet on rats. Long Evans male and female rats were fed a diet containing phytoestrogens 600 µg/g diet (phyto-600) or a phytoestrogen-free diet. According to Lund *et al.*, 2001 *(247)*, the phyto-600 diet contained soy and the phytoestrogen-free diet contained fish meal. Diets were similar in protein, fat, amino acid, mineral, and vitamin content. Rats were fed the diets prior to mating and throughout pregnancy and lactation. On weaning at 30 days of age, 10–14 F_1 offspring/sex/group received the same diet as their dams. **[The number of litters from which the offspring were obtained was not stated.]** Body weight and feed and water intake were measured in offspring at 50 days of age. At 75 days of age, anxiety was assessed in the offspring using an elevated plus-maze. Data were analyzed with ANOVA and Bonferroni post hoc comparisons. Body weights were significantly lower in males **[4%]** and females **[5%]** fed the phyto-600 diet, despite a significant increase in feed intake in the phyto-600 males. Water intake was significantly higher in males and females fed the phyto-600 diet. A total of 10–14 rats/sex/group completed the maze test in all groups except the phyto-600 females. Only 7 females of the phyto-600 group completed the test, because 5 animals fell or jumped off the open arms. The phyto-600 diet was observed to have behavioral effects, as noted by an increased percentage of time spent in open arms **[~60 compared to 30% in the phytoestrogen-free group]** and increased numbers of entries into open arms **[~45 compared to 25% of entries]** by both males and females. The study authors concluded that phytoestrogens in soy caused marked reduction in anxiety in rats.

Strengths/Weaknesses: A strength of this study was that the authors used the dietary route of exposure, which is a relevant route, and the concentration of phytoestrogens was relevant to laboratory animals (commercially available laboratory diet with high phytoestrogen levels; 600 µg/g diet). Rats were exposed to phyto-600 or phytoestrogen-free diets through critical windows of development (pre-mating, mating, gestation, lactation, and into adulthood) to monitor the long-term effect of these different diets. Circulating levels of phytoestrogens using this dosing paradigm were reported in previous publications by these authors. Statistical analyses were appropriate. A weakness is that this study used a single dose level of phytoestrogens (phyto-600), which does not allow for an evaluation of dose-response relationships. It is not clear whether the authors controlled for litter effects (i.e., consideration of the litter of origin when assigning pups to different endpoints or use of the litter as

the unit for statistical analysis). Fetuses within the same litter exposed to phytoestrogen *in utero* are not independent samples. Body weights and feed and water consumption were monitored for only 3 days at 50 days of age. Body weights at PND 75, the time of maze testing, were not reported. Rats were tested only once for 5 minutes in the plus-maze.-this is standard

Utility (Adequacy) for CERHR Evaluation Process: Although a relevant route of exposure was used and the concentration of phytoestrogens used are relevant the use of a single dose obviates determination of dose-response relationships and thus this report is of limited useful in the CERHR evaluation process.

Lund et al., 2001 ***(231)***, supported by the National Science Foundation and Brigham Young University, examined the effects of lifelong phytoestrogen exposure on visual spatial memory in rats. In the first 2 parts of the experiment, Long Evans rats were fed either a soy-based diet containing phytoestrogens 600 µg/g diet (phyto-600) or a phytoestrogen-free diet during pregnancy and the lactation period. **[The number of dams treated was not specified.]** According to Lund *et al.*, 2001 *(247)* the protein source for the phytoestrogen-free diet was fish meal. Diets were similar in protein, fat, amino acid, mineral, and vitamin content. Ingestion of the phyto-600 diet was said to result in blood phytoestrogen levels similar to Asians consuming high amounts of soy. The phytoestrogen-free diet was representative of Western populations that consume very little soy. Offspring were weaned 30 days following birth and given the same diets as their mothers. The diet was given ad libitum until 50 days of age when feed was limited to maintain the rats at ~90% of normal body weight. Beginning at 57 days of age, the rats (n=13–16/sex/group) were tested for their ability to retrieve a food reward from an 8-arm maze. Once the rats mastered the task they were tested for 10 days (1 trial/day). Following completion of the test, rats were killed and brains were collected for measurement of calbindin and cyclooxygenase-2 levels by Western blot. The first part of the experiment examined males, and the second part examined females. The 2 parts of the experiment were conducted independently of one another but at the same time intervals, using the same procedures. Data were analyzed by 1-way ANOVA and multivariate ANOVA.

Males fed the phyto-600 diet required significantly more trials to master the 8-arm maze than males fed the phytoestrogen-free diet (13 compared to 11 trials). In contrast, females fed the phyto-600 diet mastered the maze in significantly fewer trials compared to females fed the phytoestrogen-free diet (11.5 compared to 13.5 trials). No effect of diet on working memory was detected **[data not shown]**. In the frontal cortex, high levels of phytoestrogens corresponded with decreases in calbindin and increases in cyclooxygenase-2 in males only. Calbindin-protein expression in the frontal cortex but not hippocampus was significantly lower in males fed the phyto-600 compared to the phytoestrogen-free diet. Cyclooxygenase-2 protein expression was significantly lower in the frontal cortex of males fed the phytoestrogen-free compared to the phyto-600 diet. No effects of diet on calbindin and cyclooxygenase-2 protein expression in brain were detected in female rats. The study authors stated that calbindin protects against neurodegenerative diseases and apoptosis through regulation of intraneuronal calcium. Cyclooxygenase-2 is an inflammatory protein associated with key steps in the development of Alzheimer's disease.

In the third part of the experiment, male and female rats were exposed to the phyto-600 diet from gestation through adulthood, and the 8-arm maze testing (described above) was conducted in 11–13 rats/sex/group, as described for the first and second parts of the experiment. One trial/day was conducted over a 10-day period. Following completion of the maze testing, half the rats were randomly selected to receive the phytoestrogen-free diet and the other half continued to receive the phyto-600 diet ad

libitum for 15 days. For the next 10 days, feed was restricted to maintain the rats at ~90% of normal body weight. When rats were reduced to 90% of normal body weight, they were tested for 15 days (1 trial/day) using an 8-arm maze in which only 4 arms contained a food reward (n=5–7 rats/sex/group).

Males fed the phyto-600 diet mastered the maze in significantly fewer trials compared to females fed the same diet (8 trials in males compared to 12 trials in females). When trial results were averaged over 2-day periods, males had significantly more correct arm entries than females in 3 of the 5 periods. Significant diet-related and sex-related effects were observed in the 4-arm maze test that was conducted after half the animals continued to receive the phyto-600 feed and the other half were switched to the phytoestrogen-free feed. Data were presented as the number of correct arm entries and were averaged over a 3-day period. In general, females maintained on the phyto-600 diet performed better than females switched to the phytoestrogen-free diet. In contrast, performance was better in males switched to the phytoestrogen-free diet compared to males that continued to receive the phyto-600 diet. In rats switched to the phytoestrogen-free diets, males performed better than females. During the last 2 sets of 3 trials, males switched to the phytoestrogen-free diet made significantly fewer reference errors (entering an unbaited arm) than males maintained on the phyto-600 diet. There was no effect on working memory (re-entering a baited arm) or working/reference (re-entering an unbaited arm). Both sexes maintained on the phyto-600 diets completed the task in significantly less time than animals switched to the phytoestrogen-free diet.

Isoflavones were detected in several brain regions of F_0 males fed the phyto-600 diet in adulthood. Concentrations in frontal cortex were about 2 orders of magnitude higher than in hippocampus. According to study authors, both of those brain regions are critical for visual spatial memory. Additional details about brain levels of phytoestrogens are provided in the toxicokinetics discussion in **Section 2**.

The study authors concluded that dietary phytoestrogens caused a reversal in sexual dimorphic expression of visual spatial memory.

Strengths/Weaknesses: This study had several strengths and was well designed in that the authors exposed rats to phyto-600 or phytoestrogen-free diets through critical windows of development (pre-mating, mating, gestation, lactation, and into adulthood) to monitor the long-term effect of these different diets. Furthermore, by switching some adults to the alternate diet, the authors could examine the reversibility of diet-related differences in maze performance. Diet is a relevant route of exposure, and the dietary concentration of phytoestrogens was relevant to both laboratory animals (commercially available laboratory diet with high phytoestrogen levels; 600 µg/g diet) and humans (reported plasma levels in rats were similar to Asians consuming large amounts of soy protein per day). Maze testing and analysis were conducted blind to treatment group. Measurements were made to determine the levels of phytoestrogens in different brain regions, calbindin in the frontal cortex and hippocampus, and cyclooxygenase-2 in the frontal cortex. Circulating levels of phytoestrogens using this dosing paradigm were reported in previous publications by these authors. A weakness of this study was that it used a single dose level of phytoestrogens (phyto-600), which does not allow for an evaluation of dose-response relationships. It was not clear whether the authors controlled for litter effects (i.e., that they considered the litter of origin when assigning pups to different endpoints or used the litter as the unit for statistical analysis). Fetuses within the same litter exposed to phytoestrogen *in utero* are not

independent samples. Body weight data were not presented in the manuscript, although the authors noted that body weight effects were the same for males and females and should not have accounted for sexually dimorphic differences in maze performance. In the Methods section, the authors stated that the phyto-600 diet contained phytoestrogens 600 µg/g diet, whereas in the legend to Table 1, it was stated that pups were exposed to phytoestrogens 300 µg/g diet from conception until 50 days of age. The reason for this difference was unclear. The authors also mentioned that plasma levels of phytoestrogens were determined but did not present these data. Brain levels of phytoestrogens were only measured in males. Phyto-600 diet caused opposing effects on visual spatial memory in males and females, yet in examining the frontal cortex, no diet-related differences in calbindin or cyclooxygenase-2 were detected in females. It would have been useful to measure phytoestrogen levels in brain regions in females to determine if phytoestrogen levels were similar to levels seen in males. The diet change in adulthood from phyto-600 to phytoestrogen-free diet showed reversal of effects (male performance improved, whereas female performance declined); however, the authors did not state whether a complete reversal was achieved. This point is important because a complete reversal of effects would suggest that *in utero*/lactational exposure did not cause long-term effects on maze performance. Lastly, the authors did not show the data for working memory, which was not altered by diet in either males or females. The rationale for the choice of calbindin and cox-2 as markers is also not clear.

Utility (Adequacy) for CERHR Evaluation Process: This report is of no utility in the CERHR evaluation process.

Taylor et al., 1999 ***(724)***, supported by the National Science Foundation and Brigham Young University, examined the effects of a phytoestrogen-containing diet on fetal calbindin levels in the medial basal hypothalamus and preoptic area of the brain. Pregnant Sprague Dawley rats were fed a phytoestrogen-containing diet (n=4) or phytoestrogen-free diet (n=2) on GD 0–20 (day of plug=GD 0). The phytoestrogen diet (phyto-200) contained phytoestrogens 200 µg/g diet (genistein 95 µg/g, glycitin 17 µg/g, and daidzein 82 µg/g). **[The protein source of the phyto-200 diet was assumed to be soy; the protein source for the phytoestrogen-free diet was not given.]** Diets contained the same percentages of protein, fat, and carbohydrates. Rats were killed on GD 20. Fetuses were sexed, weighed, and evaluated for anogenital distance (n=27–30/sex in the phyto-200 group and 15–16/sex in phytoestrogen-free group). The medial basal hypothalamus and preoptic area brain regions were collected from ≥5 fetuses/sex/litter/dietary group and pooled according to sex and dietary group. Medial basal hypothalamus and preoptic area calbindin levels were measured by Western blot. Data were analyzed with ANOVA, followed by pair-wise Tukey comparison. No significant effects of diet were detected on feed or water intake during gestation or on litter size **[data not shown]**. No effects of diet on anogenital distance and fetal body weight were detected. Calbindin protein expression in the medial basal hypothalamus and preoptic area was significantly higher **[by ~40%]** and more similar to male values in females fed the phytoestrogen-free compared to the phyto-200 diet. Diet had no effect on calbindin protein expression in males. The study authors concluded that the data have far-reaching implications regarding possible influence of dietary phytoestrogens on fetal medial-basal hypothalamus and preoptic-area calbindin levels.

Strengths/Weaknesses: A strength was that animals were exposed in the diet, which is a relevant route of exposure. Diets were analyzed by HPLC to determine concentrations of the phytoestrogens

genistein and daidzein. Western blot data were obtained from six immunoblots. Specificity of the calbindin antibody was verified by demonstrating a lack of binding to other calcium-binding proteins. After the chemiluminescence assay, blots were stained with India ink to ensure equal protein loading and efficient transfer. For immunohistochemistry, a negative control was included using adjacent tissue sections with pre-immune serum or a buffer-only control (without the primary antibody). A weakness was that the study included only one dietary level of soy, which did not allow for a comparison of dose-response relationships. The stability of the phytoestrogen-containing diets was not reported Medial basal hypothalamus and preoptic area of the brain were collected from ≥5 males and ≥5 females/litter and were pooled by sex and dietary group, thus suggesting that not all litters were equally represented in the samples analyzed. Blood levels of genistein/daidzein were not measured. The authors did not discuss how these data relate to human hypothalamic development.

Utility (Adequacy) for CERHR Evaluation Process: This report is of limited utility in the CERHR evaluation process. The data clearly show that exposure *in utero* to phytoestrogens ingested by dams affects brain development in females. Female embryos on the soy diet had less Calbindin in the MBH than females in the no soy group. No effect of diet was noted in males.

Weber et al., 2001 ***(236)***, supported by the National Science Foundation, examined the effects of low- and high-phytoestrogen diets on aromatase activity on medial basal hypothalamic and preoptic areas of maternal, fetal, and neonatal rats. Sprague Dawley rats were placed on a high-phytoestrogen (n=10) or a low-phytoestrogen (n=13) diet. The commercially available high-phytoestrogen diet contained phytoestrogens 603 µg/g consisting mainly of daidzin, genistin, and glycitin. According to Lephart *et al.*, 2002 *(237)*, the diet was soy-based. The isoflavone levels were below the detection limits in the low-phytoestrogen diet, which had the same levels of protein, carbohydrates, and fats as the high-phytoestrogen diet. Rats were mated at 80 days of age and remained on their respective diets throughout pregnancy and lactation. At GD 16.5 and 20.5 **[day of plug not specified]** and on PND 3.5 **[day of birth not specified]**, brains were collected from dams and offspring. At those same time points, offspring (n=16–34/sex/group/time period) were sexed, weighed, and evaluated for anogenital distance. The medial basal hypothalamic-preoptic brain area of dams (n=3–5/time period) and offspring (n=9–15 sex/group/time period) was incubated in medium with testosterone for determination of aromatase activity by a "tritiated water" method. Isoflavone levels were measured in blood of dams and offspring at each time point, and the results of the analyses are discussed in **Section 2.1.2**. Data were analyzed by ANOVA, followed by pair-wise comparison with the Newman-Keuls test.

Fetal body weights were significantly higher in the low-phytoestrogen group on PND 20.5; no offspring body weight effects were detected at the other time periods. Anogenital distance was significantly higher in low-phytoestrogen males on GD 20.5, but when normalized for body weight, anogenital distance was significantly greater in high-phytoestrogen males and females. On PND 3.5, anogenital distance was significantly longer in high-phytoestrogen females, and no statistically significant effects were observed when normalized for body weight. Phytoestrogens crossed the placenta and were lactationally transferred to pups; however, no effects of the phytoestrogen diets on brain aromatase levels in pregnant dams, fetuses, or neonates were detected. The study authors concluded that dietary estrogens did not alter aromatase activity in the medial basal hypothalamic-preoptic brain area during perinatal development.

Strengths/Weaknesses: A strength of this study was that plasma phytoestrogen levels were measured in pregnant dams (GD 16.5 and 20.5), fetuses (GD 20.5), and neonates (PND 3.5). Animals were exposed to phytoestrogens in the diet, which is a relevant route of exposure. Diets were analyzed by HPLC to determine concentrations of the phytoestrogens genistein, daidzein and glycitein (although this determination appears to have been made in previous studies). Sex, body weight, and anogenital distance were recorded in the offspring. Analysis of blood samples included internal controls to validate the assay and verify recovery. A coefficient of variation was provided to establish assay precision and, as a comparison, adult male plasma phytoestrogen levels were determined for males on the same diets. A weakness of the study is that it included only one dietary level of soy, which did not allow for evaluation of dose-response relationships. The stability of phytoestrogen-containing diets was not reported. It was not specified whether the day on which the females were sperm-positive was GD 0 or GD 1, nor was the day of delivery specified as either PND 0 or PND 1. The authors did not provide litter parameters (e.g., fertility, litter sizes, sex ratio). They also did not provide body weights at the time of randomization. It did not appear that authors controlled for litter effects (i.e., in Table 2 and Figure 1B of the study, they did not consider the litter as the unit of analysis for animals exposed during gestation and lactation). Blood samples were pooled by treatment (representing 3–5 litters per collection); therefore, variance in blood levels could not be determined (Table 1 of the study). Maternal body weights and body weight gains during gestation were not given. It is puzzling that the increases in relative anogenital distance (normalized to body weight) were transient (not different at PND 3.5). The differences in protein sources (soybeans and fish) likely contributed other differences aside from phytoestrogen levels. Odum *et al.*, 2001 *(622)* reported that different rodent diets with different amounts of phytoestrogens had centrally mediated effects on rodent sexual development due to nutritional differences between the diets rather than ER-mediated effects. The authors assessed plasma phytoestrogen concentrations by ANOVA (Table 1 of the study), despite using pooled samples and not presenting variances. The authors did not discuss how these data relate to human hypothalamic development.

Utility (Adequacy) for CERHR Evaluation Process: Due to methodological issues and lack of dose response data, this report is considered of no utility in the CERHR evaluation process.

3.4.4 Other Endpoints (Thyroid, Immune, Bone, etc.)

3.4.4.1 Rats

Chang and Doerge, 2000 ***(242)***, of the FDA, examined the effects of post-weaning and adult exposure to soy diets on thyroid function in rats. The effects of genistein added to diet were also examined, and that portion of the study is discussed in **Section 3.3**. On PND 21–190, two groups of male and female Sprague Dawley rats were fed either NIH 31, a soy-based diet reported to contain genistein and daidzein at 30 ppm each or a soy-free 5K96 feed. **[The number of rats treated was listed as 6, and it is assumed that it was 6/sex/dose as in the main part of the study.]** Microsomal thyroid peroxidase activity was determined using a spectrophotometric method to measure guaiacol oxidation. **[It is assumed that data were analyzed using 2-way ANOVA or Dunnett test as in the main part of this study.]**

Thyroid peroxidase activity was significantly reduced in rats fed the soy-based compared to soy-free diet. Thyroid peroxidase activity in the soy diet group was less than half the activity in the soy-free

diet group. The study authors noted that the effect was similar to that observed in rats fed diets containing 100 ppm genistein in the aglycone form. Thus, it was noted that the form of genistein did not affect total serum isoflavone concentrations or decrease thyroid peroxidase activity inhibition. Serum levels of genistein and daidzein were also measured and are discussed in **Section 2**. The study authors noted that consumption of isoflavones by humans could result in uptake by thyroid gland and inactivation of thyroid peroxidase.

Strengths/Weaknesses: Strengths of the study include the administration of genistein aglycone in the diet, the use of 3 dose levels, use of a soy-free diet and the measurement of genistein and daidzein contents of the feed. The rats were exposed to genistein aglycone starting *in utero* through 20 weeks corresponding to lifelong exposure. Another strength of this study was the measurement of total genistein and daidzein levels in serum using HPLC with electrospray MS, a method that generated very low detection levels (20 nM genistein in serum). Thyroid peroxidase assay procedures were validated for linearity of response versus protein, and steps to prevent loss of thyroid peroxidase activity during isolation were well described. Assuming that statistical analyses were conducted as in the main part of the study examining addition of genistein to feed, the analyses were appropriate, using 2-way ANOVA to examine dose, sex, and dose/sex interactions. A weakness of this study is that stability of genistein in the diet was not verified. Isoflavone dose levels were not reported in mg/kg bw/day, and feed consumption data were not provided however analysis of genistein levels in serum was conducted allowing estimation of exposure. There were no details as to how prepubertal pups were assigned to treatment groups and no indication that the authors controlled for litter effects. It is not clear why rats were killed at 190 days in the diet-comparison study compared to 140 days in the genistein-supplemented diet study. The authors did not include a discussion on the sensitivity of the rat model to thyroid perturbations (e.g., McClain *(725)*).

Utility (Adequacy) for CERHR Evaluation Process: This report alone is of no utility in the CERHR evaluation process. The biological relevance of a reduction in TPO activity is unclear from this study. The authors indicate that other separate analyses have revealed no changes in thyroid hormone levels, thyroid weights, or histopathology suggesting that the altered TPO activity is not adverse. It would be helpful to see other studies that assess the potential effects of dietary genistein on thyroid function.

Churella et al., 1976 ***(726)***, from Ross Laboratories, examined the effect of trypsin-inhibitor levels in soy formula on development of rats. Rat diets containing varying levels of trypsin inhibitors were prepared by using soy formulas that were not treated, were heat processed, or were heat processed and sterilized to reduce trypsin-inhibitor activity. At 21 days of age, 12 Sprague Dawley rats/group were given feed containing 235, 40, 10.4, 14.7, or 11.5 trypsin-inhibitor units/100 g feed for 3 weeks. A control group was fed a casein-based diet containing 1.5 trypsin-inhibitor units. Diets were fairly similar in protein, fat, carbohydrate, and *d,l*-methionine content. Food intake and body weights were measured over the 3-week period. Animals were then killed for measurement of final body weights and liver and pancreas weights. Pancreases were examined histologically. **[Histologic methods were not discussed, and it was not stated how many rats per group were examined.]** Data were evaluated by ANCOVA. Compared to controls, liver weights relative to body weights were significantly reduced **[by ~10%]** in rats fed diets with 40 or 11.5 trypsin-inhibitor units. **[Absolute organ weights were not reported.]** No effects were detected on body weight gain, relative pancreas weight, or pancreatic hypertrophy or hyperplasia. In the analysis of

5 infant soy formulas, 4 were found to have trypsin-inhibitor activity at <15% of the activity of soy-protein isolate used to manufacture the formulas. The study authors concluded that residual trypsin-inhibition activity is low in soy formulas and, at those levels, would be of no nutritional significance to the rats.

Strengths/Weaknesses: A strength of this study is that diets included soy-based infant formulas (at different stages of processing to heat-inactivate soybean trypsin inhibitor, soy isolate (the soy protein used to manufacture formulas), and a casein-based rodent diet. Duration of the treatment period (3 weeks) was determined in a probe study, which demonstrated this as the time of maximum pancreatic hypertrophy for rats fed unheated soy protein. Weanling rats were placed into treatment groups such that each group had similar body weight distributions. The authors calibrated the trypsin-inhibition assay, demonstrating that 1 g purified soybean-trypsin inhibitor inhibited 1.64 g trypsin. The impact of processing soy isolate on its soybean-trypsin inhibitor content was evaluated. In deriving the soybean-trypsin inhibitor inhibition curve, extract concentrations were used in a range that inhibited 20–80% of trypsin activity, as values outside this range were not linear. A weakness of this study is that there was no indication that the authors controlled for litter effects. The trypsin used to analyze soybean trypsin-inhibitor activity was 52% pure. Dose-response could only be evaluated for soybean trypsin-inhibitor activity. Limited endpoints were examined in the study. Histology procedures were not described. Weanling rats were used in this study, so critical developmental stages during the neonatal period were not evaluated.

Utility (Adequacy) for CERHR Evaluation Process: This report has no utility for the CERHR evaluation process. While these experiments are helpful for examining soybean trypsin-inhibitor activity, it is difficult to judge the effects of the different formulas as the authors supplemented the lyophilized formulas with numerous other components (carbohydrate, fat, minerals, vitamins, and methionine) when preparing the diets. Numerous additional methodological limitations of the study, as identified above, further prohibit the utility of this report for the evaluation process.

Daly et al., 2007 ***(727)***, supported by the Howard Hughes Medical Institute and Joint Institute of Food Safety and Nutrition, investigated the effects of dietary soy isoflavones on early stage colon cancer in various ages of female rats. Three ages of rat and two diets were used in the experimental design (6 groups, n=7 rats/group): young (one month old), mature (11 months old) and aged (22 months old). $F_3$44 female rats were obtained from NIH (Bethesda, MD) and fed either an AIN-93 control diet, or a soy isoflavone experimental diet (4 g Novasoy/kg AIN-93 diet; analysis revealed a concentration of 2.6 mg isoflavone/gram diet) for the duration of the experiment. The concentration of soy was based on the effective dose used in a prostate chemopreventative study. Bedding was not used **[purity of the Novasoy not stated]**. One week after being assigned to experimental diets, all rats were given a single 20 mg/kg sc injection of azoxymethane (AOM). The rats were weighed daily until body weight was restored and then weighed weekly for the remainder of the study. At the start of week six, food consumption was measured over a 3-day period. Fifteen weeks after AOM injection (16 weeks on diet), the rats were killed, blood was collected and the following tissues were saved in formalin, and examined for histopathological changes: uterus, ovary, mammary, liver, kidney and two sections of colon; in addition, a colon mucosal layer sample (scraping) was fixed in RNA*later*. An additional colon section was stained with methylene blue and the total number of Aberrant crypt foci (ACF) and ACF multiplicity were quantified for each animal. Serum estrogen levels were quantified

using the Ultra-Sensitive Estradiol RIA kit. Colonic ERβ mRNA was quantified by RT-PCR using Estrogen Receptor Type 2 Assay-on-Demand kit. ERβ expression was measure relative to 18S rRNA expression. ERβ protein was evaluated used immunohistochemistry. One-way analysis of variance (ANOVA) was performed for all data and two-way ANOVA was completed to compare diet and age for all data. The general linear models (GLM) procedure for unbalanced designs was used due to deaths during the experiment. Multiple comparison Student-Newman-Keuls test was performed when the ANOVA showed significance.

Throughout the experiment and for all age groups, the rats fed the soy diet had significantly lower body weights than those fed the control diet; there was no effect of diet on food consumed. All rats fed the control diet survived until the end of the experiment, one mature rat and three old rats fed the soy diet died after AOM treatment. Young rats had a significantly greater number of ACF compared to mature and old rats (average 53, 26, and 22 for young, mature, and old rats, respectively); there was no significant effect of diet on ACF number or multiplicity. Serum estradiol concentration (pg/mL) decreased with age for the animals on control diet but increased with age for animals on the soy diet - all three soy groups had similar concentrations compared to the young control rats. Isoflavones were not detected in the serum of control rats; although there were higher levels of genistein than daidzein in the soy diet, serum levels of genistein and daidzein were similar within each soy-diet age group. The ratios of uterine weight to body weight were similar among all age groups fed the control diet, but increased with age in rats fed the soy diet. There were no adverse histological effects attributable to the soy diet. There were no significant differences in ERβ mRNA expression in the colonic mucosa, in ERβ protein staining intensity, or in the distribution of ERβ positively stained cells in colonic crypts among age or diet groups. Young rats had a greater density of the normal crypts lining the colonic mucosa compared to mature or aged rats; control fed rats had significantly higher crypt density compared to the soy-fed rats. Soy-fed rats also had lower numbers of epithelial cells, more lamina propria between crypts and higher numbers of stromal cells, indicative of crypt loss/crypt dropout.

Authors' conclusion: The authors report an unexpected interaction between age, gender, dietary isoflavones and the acute effect of a colon carcinogen (azoxymethane), resulting in increased immediate sensitivity of aged animals and significant persistent morphological mucosal changes. No beneficial effect of isoflavones on colonic ACF development was observed in any age group of female $F_3$44 rats.

Strengths/Weaknesses: Strengths include the use of a semi-purified diet; NovaSoy extracts and diets were analyzed for isoflavone concentrations. Route of administration is relevant to human exposures and serum level of isoflavones were measured. Use of a standard animal model and carcinogen is reasonable, as is the use of different ages. Weaknesses include the administration of the carcinogen after initiation of the dietary manipulations. Effects of the diets on carcinogen PK/PD could confound data interpretation (if not already known not to have such effects).

Utility (Adequacy) for CERHR Evaluation Process: The study did not include any *in utero* or prepubertal exposures; youngest group was one month of age. Study is of no utility for the evaluation process but useful in defining age of exposure as a possible variable in determining response to soy exposures on colonic ACF formation. There are few studies in this area but raises possibility of detrimental effects of soy exposure on colon cancer and identifies an area for further investigation.

Douglas et al., 2006 ***(728)***, supported by Tommy's the Baby Charity, examined whether the cardiovascular benefits of soy protein are attributable to soy-associated isoflavones by evaluating cardiovascular function in animals fed throughout life with an isoflavone rich soy protein diet or the same diet in which the soy was replaced with isoflavone-depleted soy protein. Male and female Wistar rats (Harlan, UK, 100-120 days of age) were fed for 10 days prior to mating and throughout pregnancy and lactation on either 1) a balanced diet containing soy protein, rich in naturally occurring isoflavones (260 mg/kg) or 2) the same diet in which the soy was replaced with soy protein from which the isoflavones had been eluted (40 mg/kg), there were 10 rats per group. The concentrations of isoflavones in the high-isoflavone diet were designed to mimic those in soy protein-based rodent chow. At birth, litters (n=5 or 8) were reduced to six to eight pups and, when possible, to equal numbers of males and females. **[Bedding not described]** Offspring were fed the same diet as the dam and sire. Food intake and animal weights were recorded daily during pregnancy and weaning, then weekly from 22 to 180 days of age. Blood pressure, heart rate and activity were assessed by radiotelemetry in the same animals subsequently used for the study of isolated artery function. Oestrus cycle was determined in female offspring via daily vaginal smears for 14 consecutive days prior to telemetry surgery, at 3 months and again at 6 months. During blood pressure and heart rate recording, the stage of oestrus was established indirectly from remotely recorded activity data. Vascular function was assessed in 3- and 6-month-old offspring; one male and one female from each litter were studied. Blood samples for determination of fasting concentrations of total cholesterol, triglycerides and high-density lipoproteins were obtained. Third-order mesenteric arteries and the thoracic aorta were placed in ice-cold physiological saline. Third-order mesenteric arteries were mounted on a pressure myograph and changes in diameter to incremental pressure steps were measured over the range of 20-100 mmHg at 2-minute intervals. Arterial responses to the same pressure range were assessed, but in the absence of active tension. Third-order branches of the mesenteric artery were mounted on a small vessel myograph; concentration-response curves to noradrenaline (NA), endothelin, and acetylcholine and aqueous nitric oxide (NO) in NA pre-constricted vessels were determined. Two transverse rings of the thoracic aorta, 2mm in length were mounted in a tissue bath, maintained in PSS, and a force of 5 mN (1 g) was applied. Concentration responses were determined to phenylephrine, and to acetylcholine and aqueous NO after preconstruction with phenylephrine. The passive diameter-force relationship was determined over three cumulative stretches of 2 min duration following equilibration in CA^{2+} free PSS. Blood samples were obtained from non-fasted littermates of animals used for the vascular protocols. Plasma was acidified with acetic acid (final pH 4.9). A calibration curve was produced by spiking control samples of human plasma with a known concentration of each isoflavone and HPLC was performed. Radio-telemetric data were assessed over 1 week and reported as the mean value over 12 h night (active) and day (inactive) periods. Day and night averages were analyzed by repeated measures analysis of variance (RM ANOVA). Relaxation was calculated as a percentage of pre-constrictor tone. Statistical analyses of comparisons across the entire concentration-response were assessed by RM ANOVA. pEC_{50} was calculated by fitting raw data to sigmoidal logistic curve and analyzed by ANOVA. Vaginal smear data was assessed by χ-square test. All other data were assessed by ANOVA. Where no significant difference between sexes was found, data for males and females were combined. A p-value of < 0.05 was considered statistically significant.

Dietary isoflavones did not affect maternal body weight during pregnancy. Offspring body weight was unaffected by diet. Daidzein, equol and genistein were detected in plasma from male rats fed an isoflavone rich diet. Isoflavones were not detectable in plasma from rats fed the low isoflavone diet.

There was no effect of sex or diet on plasma concentrations of total or HDL cholesterol or triglycerides in 3- or 6-month-old offspring. No significant effect of isoflavone consumption on blood pressure or heart rate was observed. At 3 months, but not at 6 months, male and female rats eating the isoflavone rich diet was significantly more active during the dark period (n=6). Isoflavone consumption did not affect activity during the light (inactive) period. There was no effect of diet on the estrous cycle (n=8 or 10). There was no effect of either diet or sex on mesenteric artery diameter at either 3- or 6- months of age. Acetylcholine-mediated endothelium-dependent relaxation was not different between the two groups at 3 months, but after 6 months animals fed the isoflavone rich diet exhibited increased sensitivity to acetylcholine (n=7-10). The high isoflavone diet had no effect on endothelium-independent relaxation to aqueous NO, or on responses to noradrenaline or endothelin at 3 or 6 months. Vascular distendability was unaffected at 3 months, but at 6 months mesenteric arteries from females (but not males) fed the high isoflavone diet were significantly more distensible than arteries of the females fed low isoflavone diet. There was no significant effect on wall thickness. There was no significant difference in the concentration-response curve or the pEC_{50} to phenylephedrine, acetylcholine, NO, or passive stretch-induced aortic tension for the aorta of the two groups at either time point.

Authors' conclusion: The isoflavone content of soy protein has no influence on blood pressure in healthy rats fed a diet based on soy protein, but influences small artery function.

Strengths/Weaknesses: Strengths of the study include dietary exposure, inclusion of 2 dose levels of isoflavones, use of an appropriate period of exposure, control for litter, and determination of isoflavone levels in blood. The study was focused on the assessment of cardiovascular function and used appropriate endpoints to evaluate this. The study was not designed to evaluate reproductive and developmental parameters, the only endpoint evaluated was the estrous cycle.

Utility (Adequacy) for CERHR Evaluation Process: This study has limited utility for the evaluation. The absence of effects of exposure to dietary isoflavones during development on cardiovascular function are useful data.

Mardon et al., 2008 ***(729)***, **[support not indicated]**, examined the specific effects on bone health and status of a perinatally and/or lifelong exposure to soy isoflavones (IFs) during aging. Sixty female Wistar rats (3 months old) were obtained from Institut National de la Recherche Agronomique (INRA; Clermont-Ferranf/Theix, France) and were randomly divided into two groups (n=30 females per group): one group was fed a standard diet devoid of soy proteins (replaced by casein); the second group was fed the same food enriched with IFs. The powdered soy IF concentrate contained 348 mg/g as total IF (159 mg genistein, 156 mg daidzein, 33 mg glycitin) this resulted in an IF intake of ~ 40 mg/kg body wt/day. **[bedding was not described]** Body weight and feed intake were measured weekly. After a 30-day diet acclimation period, females were allowed to mate. F_0 females remained on the same regimen during the entire gestation and lactation periods. F_1 pups were weaned at 21 days of age (PND 21); at weaning the female offspring were assigned to one of four nutritional groups: one half of the pups from the control mothers were fed the control diet (C-C) and the other half were given the IF diet (C-IF); one half of the pups from the IF-treated mothers were fed the control diet (IF-C) and the other half were given the IF diet (IF-IF). 10 F_0 females per group were killed at 2 months postpartum, and 10 F_1 females were killed per group at each of the following time points: 2, 3, 6, 12, 18, and 24 months after birth. 48 hours before death, the body composition was estimated by dual-energy x-ray

absorptiometry - body fat mass, body lean mass and whole body bone mineral content (BMC) were measured. On the day before death, 24-hour urine samples were collected to assess deoxypyridinoline (DPD) as a marker of bone resorption; DPD was determined by competitive radioimmunoenzymatic assay. On the day of death, blood samples were collected (heparin and aprotinin); plasma was measured for osteocalcin (OC) - a marker of osteoblastic activity - by radioimmunoassay (RIA). Uterine horns were weighed and both femurs were collected for mechanical testing and bone mineral density (BMD) measurement. The total right femur BMD (T-BMD), the BMD of the distal femur metaphyseal zone (M-BMD), and the BMD of the distal femur diaphyseal zone (D-BMD) were assessed by DEXA(?). The length of the left femur and the mean diameter of the femoral diaphysis were measured with a precision caliper. Femur failure load was determined by a 3-point bending test. Analysis of Variance was performed when data were sampled from populations with equal variance. When significant, the Student-Newman-Keul's multiple comparisons test was used. If not parametric, a Kruskall-Wallace test was performed; if it indicated significance, the Mann-Whitney *U* test was used. The level of significance was set at $P<0.05$ for all statistical tests.

F_0 generation: there was no effect of treatment on body weight, fat mass, or bone parameters; The IF-supplemented mothers had a statistically significant lower uterine weight compared to the control group. Plasma OC concentration was similar to that of the age-matched F_1 generation. DPD urinary excretion was much higher than the 3-month and 6-month F_1 generation rats. *F_1 generation*: All the rats that had been exposed to IF (IF-C, C-IF and IF-IF groups) had a statistically significant increase in body weight compared to the controls (C-C).

From 6 to 18 months, uterine weight was higher (statistically significant) for the IF-C and IF-IF groups compared to the C-C and C-IF groups; this difference disappeared at 24 months. There was no difference between groups for the age at which peak bone mass was achieved. BMD stabilized approaching 24 months for the C-C and C-IF groups, but it continued to increase for the IF-C and IF-IF groups. At 24 months, the animals from IF-fed mothers exhibited a higher BMD than the rats from control mothers (C-C and C-IF); this was statistically significant for the T-BDM and the M-BMD, but not D-BMD. After 12 and 18 months, a statistically significant decrease in T-BDM, D-BDM and M-BDM was observed in the C-IF group. Femoral size was similar in all groups (data not shown). A progressive increase in the femoral failure load was recorded from 2 to 6 months in all animals. A steady state was reached at 6 months through 24 months in C-C and C-IF groups; however, for IF-C and IF-IF groups, bone strength continued to improve with age. There was no consistent difference between groups for plasma OC concentration or DPD urinary excretion.

Author's conclusion: This experiment demonstrates that rats born from mothers exposed to IFs during pregnancy and lactation exhibited improved total and metaphyseal femoral BMD, as well as bone strength at 24 months of age. The data suggest early programming events associated with phytoestrogen intake could be involved in bone mass variations during aging, since perinatal exposure to soy IFs leads to significant increases in bone status in later life.

Strengths/Weaknesses: Dietary exposure, appropriate period of exposure, evaluated multiple endpoints appropriate for bone health, and adequately powered for the assessment. Method of assignment at weaning were not clear and no assessment of isoflavone exposure. Litter effects were not taken into account.

Utility (Adequacy) for CERHR Evaluation Process: This study has limited utility for the evaluation. The data are useful for the assessment of effects of soy on bone.

Ronis et al., 1999 ***(730)***, supported by the USDA, evaluated the expression of CYP3A and CYP2B enzymes in male Sprague Dawley rats exposed to soy protein isolate during development. Parental rats were placed on AIN 93G diet in which soybean oil was replaced by corn oil. The protein source in the diet was casein for 1 group and soy protein isolate for the other group of parental animals. After several weeks on the diets, animals were mated and females were maintained on their assigned diets through pregnancy and lactation. Litters were adjusted at birth to 5 males and 5 females. Male pups were weaned to their dam's diet. Genistein consumption **[age unspecified]** was estimated at 19.3 mg/kg bw/day. On PND 65, males from each diet group received either dexamethasone or corn oil vehicle by gavage for enzyme induction. Livers were removed the next morning and microsomes were prepared using differential ultracentrifugation. Assays of CPY3A activity were performed for erythromycin N-demethylase, ethylmorphine N-demethylase, testosterone 6β-hydroxylase, lithocholic acid 6β-hydroxylase, and corticosterone 6β-hydroxylase. CYP2B was assessed by the activity of pentoxyresorufin *O*-dependentylase. Western blotting was used to measure microsomal CYP3A and CPY2B1 apoproteins and Northern blotting was used for analysis of several isozymes of *CYP3A* as well as *CYP2B1* mRNAs. Statistical analysis was performed using 2-way ANOVA followed by Student-Newman-Keuls test. Most comparisons were performed using rats from 4–6 litters/group. Body weight was 12% lower in animals fed soy protein isolate than animals fed casein. There were no diet-related differences in absolute or relative liver weight. In rats not receiving dexamethasone, corticosterone 6β-hydroxylase was increased 9% by soy protein isolate, but other CYP3A activities were unaffected. In rats receiving dexamethasone, all CYP3A activities except lithocholic acid 6β-hydroxylase (CYP3A18) were increased by soy protein isolate. Pentoxyresorufin *O*-dependentylase was increased by soy protein isolate in rats induced with dexamethasone. Western blotting showed an increase in CYP3A apoprotein in soy protein isolate-fed rats that received dexamethasone, and Northern blotting showed an increase in mRNA for *CYP3A2* in these animals. No effects of diet on *CYP2B1* mRNA and apoprotein were detected. The authors concluded that soy protein isolate increased expression of CYP3A2, particularly in dexamethasone-induced animals. They postulated that some of the variability in human neonatal hepatic CYP3A enzyme activity may be related to dietary intake of soy infant formula.

Strengths/Weaknesses: Strengths are the use of AIN 93G chow with soybean oil replacing corn oil resulting in a feed with expected low phytoestrogen content, the use of several methods to analyze CYP induction (enzyme activities, mRNA levels, and apoprotein levels), determination of cross reactivity of antibodies for Western blots, and use of the litter as the experimental unit in assays. Weaknesses were the lack of determination of phytoestrogen content of chow, the use of different protein sources in chow, the failure to specify the length of time on diet prior to breeding and the method of selection of male pups from each litter, the examination of only males, the small number of animals per group, the lack of indication of when/if feed consumption was monitored or whether feed consumption was similar between groups (a difference may have affected body weight, which was decreased in rats consuming soy protein isolate), lack of a loading control for Western blots, and lack of indication how pooling of liver microsomes was accomplished.

Utility (Adequacy) for CERHR Evaluation Process: This report is of no utility for the CERHR evaluation process.

Ronis et al., 2004 ***(731)***, supported by USDA, evaluated hepatic CYP3A in weanling male Sprague Dawley rats exposed during pregnancy and lactation to dietary soy protein isolate. Pregnant dams were obtained on GD 4 and fed AIN 93G diets in which soybean oil was replaced with corn oil. In 1 group of dams, the dietary protein source was casein and in another group, the dietary protein source was soy protein isolate (n=7–9/group). The soy protein isolate diet contained isoflavones at 430 mg/kg feed (genistein 276 mg/kg feed and daidzein 132 mg/kg feed). At birth, litters were adjusted to 5 males and 5 females. Male pups were weaned on PND 21 to their dam's diet. On PND 25, male pups were given corn oil vehicle or the CYP3A inducers dexamethasone or clotrimazole by gavage (n=1 pup/treatment/litter) or were untreated (2 pups/litter). Livers were removed the next morning and microsomes were prepared using differential ultracentrifugation. PND 25 pups in the soy protein isolate group were estimated to have consumed dietary isoflavones at 65 mg/kg bw/day. Hydroxylation of testosterone and midazolam were measured in microsomal preparations as estimates of CYP3A mono-oxygenase activities. CYP3A apoproteins were estimated by Western blot, and *CYP3A* mRNAs were estimated by Northern blot. CYP3A1 heterologous nuclear RNA was measured using RT-PCR. Statistical analysis was performed using *t tests* or 2-way ANOVA followed by Student-Newman-Keuls test; most groups contained rats from 7–9 litters. **[The Expert Panel notes that legends for several figures indicate that some groups contained only 4 or 5 rats.]**

Dietary soy protein isolate resulted in the presence of CYP3A apoprotein in hepatic microsomes, whereas casein-fed animals had undetectable CYP3A apoprotein. The soy protein-associated increased levels of CYP3A were further enhanced by both of the CYP3A inducers. *CYP3A2* mRNA and hydroxylation of midazolam (particularly 1-hydroxylation) were also increased by soy protein isolate in untreated pups. This difference in midazolam hydroxylation was not observed among pups treated with the corn oil vehicle; however, formation of the 1,4-dihydroxylated product was decreased by consumption of soy protein isolate. No diet-related alteration in CYP3A1 heterologous nuclear RNA was detected. The authors concluded that the increase in CYP3A activity associated with feeding soy protein isolate might result in altered metabolism of medications by infants on soy formula.

Strengths/Weaknesses: This report shares several features and, therefore, strengths and weaknesses, with the previous report *(730)*. In addition, there was no indication of how phytoestrogen content of soy protein isolate was determined (presumably by company that supplied it). Feed consumption presumably was monitored but there was no indication of how often, and isoflavone intake was only estimated for one time point (PND 25). The corn oil vehicle appeared to have an effect, at least on midazolam metabolism.

Utility (Adequacy) for CERHR Evaluation Process: This report is of no utility for the CERHR evaluation process.

Ronis et al., 2006 ***(732)***, supported by USDA and the Solae Company, evaluated the effect of dietary soy protein isolate during development on hepatic CYP3A1 and CYP3A2 in prepubertal Sprague Dawley rats. Pregnant dams were obtained on GD 4 and fed AIN 93G diets in which soybean oil was replaced with corn oil. In 1 group of dams, the dietary protein source was casein and in another group, the dietary protein source was soy protein isolate. At birth, litters were adjusted to 5 males and 5 females. Dams continued on their assigned diets until PND 15 at which time 5 of 11 casein-fed litters were switched to the soy protein isolate diet, and 4 of 7 soy protein isolate-fed litters were switched to the casein diet. Food pellets were added to the cage bottoms to facilitate direct consumption by pups. Rats were weaned

to their respective diets on PND 21, and livers were collected from the pups on PND 33. A second experiment was performed in which casein diets were used in 18 dams/litters from GD 4 until PND 15 at which time 6 litters were maintained on the casein diet and 3 litters/group were switched to the soy protein isolate diet, a diet supplemented with ethanol-washed (isoflavone-poor) soy protein isolate, a casein diet supplemented with genistein 250 mg/kg feed, or a casein diet supplemented with daidzein 250 mg/kg feed. On PND 33, livers and jejunums were collected. The unwashed soy protein isolate diet was estimated to provide pups a daily isoflavone intake of 65 mg/kg bw between PND 21 and 33. Hepatic microsomes were prepared by differential ultracentrifugation, and testosterone 6β-hydroxylase activity was assessed in males. Western immunoblotting was used to measure CYP3A1 and CYP3A2 in hepatic microsomes, and RT-PCR was used to quantify *CYP3A1* and *CYP3A2* mRNA in liver and jejunum. Statistical analysis was performed using 2-way ANOVA followed by Student-Newman-Keuls test. Testosterone 6β-hydroxylase activity was analyzed using non-parametric tests.

No increase in pup hepatic CYP3A1 and CYP3A2 mRNA was detected when soy protein isolate exposure was restricted to pregnancy and the first 2 weeks of lactation. Exposure on PND 15–33 increased CYP3A1 mRNA in males and females and increased CYP3A2 mRNA in males; exposure to soy protein isolate throughout gestation and to PND 33 increased both CYP3A mRNAs in males and females. Jejunum CPY3A1 mRNA was increased in both sexes by exposure to soy protein isolate on PND 15–33; no alterations in CYP3A2 mRNAs were detected in either sex. In males, hepatic CYP3A1 apoprotein was increased by exposure on PND 15–33 to soy protein isolate but not ethanol-washed soy protein isolate, genistein, or daidzein. CYP3A2 apoprotein was increased in males by exposure on PND 15–33 to daidzein but not by exposure to soy protein isolate or genistein. In females, CYP3A1 apoprotein was increased by exposure on PND 15–33 to soy protein isolate but not ethanol-washed soy protein isolate, genistein, or daidzein. Both ethanol-washed and unwashed soy protein isolate increased CYP3A2 apoprotein in females exposed on PND 15–33. Hepatic testosterone 6β-hydroxylase activity in males was increased by exposure on PND 15–33 to ethanol-washed or unwashed soy protein isolate or daidzein but not genistein. The authors concluded that developmental changes in CYP3A expression was not due to "imprinting" during pregnancy or early lactation and that soy isoflavones may have different effects on CYP3A induction than other components of soy protein isolate.

Strengths/Weaknesses: Strengths are the use of AIN 93G chow with soybean oil replacing corn oil resulting in a feed with expected low phytoestrogen content, the use of several methods to analyze CYP induction (enzyme activities, mRNA levels, and apoprotein levels), and determination of cross reactivity of antibodies for Western blots. *GADPH* was used as internal control for RT-PCR quantification of *CYP3A* mRNAs, but there was no indication that any of the treatments affected *GADPH* expression. Additional strengths are the examination of males and females, the cross-over experimental design to determine reversibility of effects, and the examination of effects of genistein and daidzein independently, although inclusion of these compounds in the ethanol-washed soy protein isolate chow would have been preferred. Weaknesses include the lack of determination of phytoestrogen content of the chow, the use of different protein sources, the lack of indication of how phytoestrogen content of soy protein isolate was determined (presumably by company that supplied it), the lack of indication of the purity of genistein and daidzein, failure to consider litter as the experimental unit, and the use of only 3 litters in several treatment groups. Feed consumption presumably was monitored, but there was no indication of how often and isoflavone intake was only estimated for one time point and one diet (PND 21–33 consuming ethanol-washed soy protein isolate).

Utility (Adequacy) for CERHR Evaluation Process: This report is of no utility for the CERHR evaluation process.

Seibel et al., 2008 ***(733)*****,** supported by a DFG grant, examined the influence of *in utero* and postnatal exposure to a phytoestrogen (PE)-rich diet on acute inflammation in an animal model of 2,4,6-trinitrobenzenesulfonic acid (TNBS)-induced colitis in male rats. Female Wistar rats were obtained from Janvier (Le-Genest St-Isle, France) and divided into two groups: group PDD was fed a PE-depleted diet containing less than 10 μg/g of the isoflavones genistein and daidzein, group PRD was fed a PE-enriched diet (Harlan Winkelmann) that contained 240 μg/g of genistein and 232 μg/g of daidzein **[basis of dose selection not stated, purity of the substances not stated]**. The females were mated with untreated males and maintained on their test diets during pregnancy and lactation **[bedding not described]**. After weaning, the male pups remained on the diets of their dams (n=16 pups for PDD, n=10 pups for PRD). At the age of 11 weeks, colitis was induced by TNBS: an enema of TNBS in ethanol-water was instilled rectally 7-8 cm proximal to the anus (at the splenic flexure). Three days after instillation, animals were weighed, killed, blood samples were collected and the distal 10 cm of the colon was removed and weighed. Portions of the colon were snap-frozen for mRNA preparations or fixed and embedded in paraffin for histological analysis. Silver stain and western blot analysis was conducted, Realtime RT-PCR was performed, and myeloperoxidase (MPO) activity was assessed. **[The results section discusses comparisons to a control group, but controls are not described in the methods section. The control animals appear to be solvent controls for the TNBS procedure, but the diet fed to these control animals was not explained and it cannot be determined whether each diet group had a set of controls, or if there was a set of controls that were fed a 'standard' diet. The number of control animals is not stated.]** The statistical significance of differences was calculated using either a two-way *t* test or a one-way ANOVA followed by Tukey HSD test where appropriate.

At day 3 after rectal instillation of TNBS, the animals had developed severe symptoms of colonic inflammation. Mean body weight increased in all treatment groups, but was significantly higher in the control animals; there was no significant difference in body weight gain between the TNBS/PDD and TNBS/PRD groups. Macroscopic inspection of the colon revealed colon wall thickening and the formation of mucosal ulcerations in the TNBS animals, but not in the solvent control animals. The weight of the distal colon (relative to body weight) was significantly greater in the TNBS groups on either diet - but was greatest in the TNBS/PRD group. Histological examination of H&E-stained sections of colon from TNBS-treated animals revealed localized ulcerations that penetrated into deep submucosal layers and corrosion of the mucosa at inflamed sites. The inflammatory response was also characterized by heavy cellular infiltration into and thickening of the submucosal layer. There were no clear differences in the histology of the two diet groups; control animals did not show signs of ulceration or infiltration. MPO activity was strongly induced upon stimulation with TNBS; this induction was significantly stronger in the TNBS/PRD group. Realtime RT-PCR revealed a strong induction of the mRNA expression of COX-2 after administration of TNBS (25-fold for TNBS/PDD rats and 15-fold for TNBS/PRD rats compared to controls). Western blot analysis of COX-2 protein expression revealed a similar expression pattern consisting of higher COX-2 protein for TNBS/PDD rats, but COX-2 was only marginally higher in TNBS/PRD rats. The expression of several colonic proteins was affected by administration of TNBS, e.g., 14kDa proteins were up-regulated and proteins in the 17-30kDa range were down-regulated.

Authors' conclusion: The results suggest that early-in-life exposure to PE might not protect against the development of IBD, but enhances the extent of acute inflammation in a rodent model of chemically induced colitis.

Strengths/Weaknesses: Strengths of this study are that exposure to genistein was by the oral route with dams being exposed during gestation as well as lactation. Further, mechanistic end points were evaluated. Weaknesses relate to lack of clarity about the study design, control groups were not well defined, and there was no apparent control for litter effects. Animal numbers were given for two of the tree groups (PDD=16, PRD=10). What is not clear is how many controls were used and which diet they were on, although table 3 suggests that they fed a PE-free chow.

Utility (Adequacy) for CERHR Evaluation Process: This study provides no utility for the evaluation process due to inadequate descriptions of animal numbers and control groups.

Teichberg et al., 1990 [734; 735], supported by the Public Health Service, evaluated the effects of early weaning to artificial diets on gastrointestinal maturation in rats. The marker of gastrointestinal maturation was the development of a barrier to the absorption of horseradish peroxidase, a 40-kD macromolecule. In the normal rat neonate, macromolecular absorption occurs until PND 17, after which absorption decreases and becomes blocked (referred to as closure) by PND 21. In these experiments, Wistar rat pups were distributed among dams to a density of 12 pups (males and females) per dam. On PND 17, the pups were divided into 3 groups of 4 pups each. One group stayed with the dam and was nursed, and 2 groups were given formula as their nutrition source using standard water bottles. The formulas included a protein-hydrolysate formula (Nutramigen®) and a soy formula (Isomil®). Formula group assignments were balanced for sex and weight. In the first study *(734)*, a separate weight-matched control group was constituted of pups that remained with their dams in litters of 12 until PND 21. On PND 21, "several litters" of pups were anesthetized and 15 cm loops of jejunum with intact vasculature were isolated. A solution containing horseradish peroxidase was placed into the isolated loop and left for 30 minutes. The luminal fluid was then collected and evaluated for volume. Horseradish peroxidase concentration was measured in blood to assess absorption. Intestinal loops were processed for histochemical localization of horseradish peroxidase using phase-contrast and electron microscopy. To examine the role of glucocorticoids, the first study *(734)* administered intraperitoneal (ip) hydrocortisone on PND 14 to some of the pups, and the second study *(735)* included corticosterone at 2 different concentrations in the formulas of some of the pups. The use of the glucocorticoid was based on the observation that glucocorticoids advanced intestinal closure in rats.

Both artificial diets in both studies were associated with about a 10% decrease in mean offspring weight on PND 21; however, no difference was detected in weights of the pups in the first study *(734)* if control pups were left in litters of 12 through PND 21. Pups fed either formula showed an increase in horseradish peroxidase absorption on PND 21 compared to pups that continued to be nursed by their dams. Histochemical evaluation of jejunal sections showed horseradish peroxidase confined to the brush border of the mucosa in control animals, whereas in animals given formula, horseradish peroxidase was evident within absorptive epithelial cells. The number of horseradish peroxidase-positive cells was greater in the soy formula-fed rats than the protein hydrolysate-fed rats. In the second study *(735)*, extension of the artificial feeding period to PND 26 showed intestinal closure to horseradish peroxidase absorption on PND 26 equivalent to that of control animals on PND 21,

suggesting that the artificial diets delayed but did not prevent closure. In both studies, glucocorticoid treatment prevented the delayed closure associated with artificial diets. The authors could not identify the mechanism by which glucocorticoids prevented the delayed closure associated with artificial diets. They believed that soy produced inflammatory epithelial damage, associated with eosinophil infiltration of the lamina propria, but found that low-dose corticosterone, equivalent to concentrations normally present in rat milk, prevented the delay in soy-associated intestinal closure without preventing eosinophil infiltration. They also noted that the effect of protein-hydrolysate formula in delaying closure was similar to that of soy and did not appear to be associated with inflammatory damage to the intestine. The authors pointed out that intestinal absorption of macromolecules is quantitatively less important in the human neonate than in the rat, and that intestinal closure is a less dramatic event in humans.

Strengths/Weaknesses: A strength of this study is that rat pups were fed soy formula, a relevant dosing scenario. The authors tried to balance sex and weight among the treatment groups when assigning rat pups. These studies were conducted on the last 4 days before weaning, a critical time for small intestine epithelial closure and a period when pups are sufficiently mature to feed independently and regulate body temperature. A weight-matched control group was included to account for the decreased body weights in the pups fed protein-hydrosylate and soy formula. The authors reported the endogenous peroxidase activity in pups not given horseradish peroxidase. Serum albumin levels were included as an assessment of nutritional status of the pups. The numbers of horseradish-positive crypt cells were assessed blind to treatment group. The low dose of corticosterone was equivalent to corticosterone levels in the maternal milk of unstressed lactating rats, whereas the higher dose was included for dose-response purposes. RIA was used to verify the absence of detectable levels of corticosterone in protein-hydrolysate and soy formulas in Study 2. Intestinal tissue was examined in pups from ≥3 experiments performed at different times. These examinations were conducted blind to treatment group. Controls included intestinal loops from animals not exposed to horseradish peroxidase. For these experiments, sample sizes appeared to have been sufficient, although there was no indication that the authors controlled for litter effects. A weakness is that both studies used only one dose level of soy formula, which does not allow for evaluation of dose-response relationships. Data were generated using the formula Isomil®, and it is difficult to determine whether these data apply to all soy infant formulas. There was no analysis of the isoflavone contents of the formulas and formula intake was not measured. There was no evidence that the authors controlled for litter effects or that that the litter was used as the unit of analysis. The number of pregnant dams from which the litters originated was not specified. Aside from maternal milk, removing pups from the lactating dam on PND 17 likely introduced other stress factors, which may have contributed to study outcome. The difference in horseradish absorption for pups given soy formula and soy formula + hydrocortisone was not statistically significant. Rat pups and human infants may vary in some aspects of gastrointestinal tract maturation; the authors state that human infants have immunoglobulin absorption across the placenta, which complicates studies on neonatal intestinal absorption. The significance of these data for humans is unclear, as intestinal absorption of macromolecules is less significant in humans. Controls not exposed to horseradish peroxidase showed peroxidase activity in eosinophils, neutrophils, monocytes, and erythrocytes.

Utility (Adequacy) for CERHR Evaluation Process: This report has no utility for the CERHR evaluation process because the relevance of this animal model is uncertain.

Tsuang et al., 2008 ***(736)*****,** supported by the National Science Council, examined whether ingestion of isoflavones before ovariectomy can prevent bone loss following ovariectomy. 30-day old female Wistar rats **[supplier not stated]** were adapted to a casein-based diet for one week. For each rat, food intake was recorded daily and body weight was recorded weekly throughout the study. The rats were randomly assigned to 4 groups (n=6 rats/group): Group A (sham surgery) and Group B (ovariectomized) were fed the casein-based diet for the entire 3-month study; Groups C and D (both ovariectomized) were fed an isoflavone-rich **[isoflavones not described]** diet (25 g soy isoflavone/rat/day) for one month before surgery. After ovariectomy, the rats in Group C were switched to control diet and the rats in Group D continued on the isoflavone rich diet for an additional two months. **[There are results for bone ash for rats at 1 month, but this is not described in the methods section]** Blood samples were collected before surgery for all rats. Sixty days after surgery, the rats were killed and the femur, the tibia, the thoracolumbar vertebrae and blood samples were collected. The long bones of the right limb and the lumbar vertebrae were burned and the weight of the bone ash for each bone was recorded. A histological study of cancellous bone was done on the left femur and tibia and the last thoracic vertebra; the thickness and interconnections between the trabeculae were recorded; the mean percent of porosity was measured. Serum levels of biochemical indices of skeletal metabolism were measured (ALP, AST/GOT, ALT/GPT, AMY, CRE, CA, and IP); parathyroid hormone (PHT) content in the serum was also measured. The data were evaluated by analysis of variance (ANOVA). Post hoc tests were performed with Bonferroni's test. Statistical significance was set at $P<0.05$.

The biochemical indices were similar between groups after one month (immediately before ovariectomy) and three months of treatment. At the conclusion of the study, body weight, serum creatinine, GPT, and PTH were higher than the preoperative baseline values. After ovariectomy, the bone ash of long bones was significantly lower than that of the sham control. At the end of the study, the bone mass of the ovariectomized rats was significantly lower in Group B and C, and significantly higher in Group D than Group A. The % mean bone porosity was greater in Groups B and C, and significantly lower in Group D compared to the sham control (Group A). The cancellous bone showed intervening trabecular bone with connectivity of the trabecular elements. In Group B and Group C rats, the thinning and disconnection of trabeculae was easily observed; in Group D rats, the trabeculation in the cancellous bone of the proximal femur and tibia was significantly higher than the sham controls (Group A) - the lumbar vertebrae appeared to show thickening of trabeculae with restoration of interconnections.

Authors' conclusion: Dietary isoflavones did not prevent the development of post-ovariectomy bone loss, but long-term ingestion of an isoflavone-rich diet increased the bone mineral contents after ovariectomy in young rats.

Strengths/Weaknesses: Strengths of the study include oral exposure and the evaluation of multiple endpoints appropriate for bone health. Weakness include the lack of determination of the amounts of isoflavones in the diet, and the lack of measurement of isoflavone exposure. More specific biochemical endpoints of bone health could have been used, and the methodology for the morphometric assessment of bone porosity was poorly defined. It is not clear if the number of animals was adequate for the assessments.

Utility (Adequacy) for CERHR Evaluation Process: This study has no utility for the evaluation.

3.4.4.2 Mice

Fujioka et al., 2007 *(737)*, supported by the Ministry of Health, Labor and Welfare of Japan, assessed the effects of dietary daidzein or genistein on bone formation in immature male and female mice. Five week old male and female ddY mice (Shizukosa Laboratory Animal Center, Shizukosa, Japan) were divided into 3 groups (n=8 mice/sex/group): Control (C), 0.08% genistein (G) and 0.08% Daidzein (D). The control diet was AIN-93 with corn oil substituted for soybean oil; genistein and daidzein (purity > 98%) were added to the diet at 0.08% (wt/wt) instead of sugar. This dose is almost half of that which has been shown to increase bone mineral density (BMD) in intact adult mice. Mice were pair-fed the experimental diets for 4 weeks; bedding was not used. Bone labeling of mice with a subcutaneous injection of calcein was performed 6 and 2 days before death. After 4 weeks on diet, the mice were killed, body weights and weights of the reproductive organs, thymus, kidney, liver, and spleen were measured, and the right and left femurs were collected. The BMD of the entire body and lumbar spine (L4 - L6) were measured using a PIXImus densitometer; the BMD of the femur was calculated using the bone mineral content (BMC) of the measured area. Histomorphometry was performed on a femoral midshaft section; mineral apposition rate (MAR), mineralizing surface/bone surface (MS/BS), and bone formation rate/bone surface (BFR/BS) on the periosteal surface were measured by calcein double labeling. Plasma genistein, daidzein and equol were analyzed using time-resolved fluoroimmunoassay (TR-FIA). The significance of the differences was determined by 1-factor analysis of covariance and Fisher protected least significant difference test. Body weight was used as covariate in the analysis of tissue weight and BMD to adjust for possible confounding. Results of the experiment are presented in **Table 152**.

Table 152. Effects of Daidzein or Genistein on Organ Weights and Bone Formation in Immature Mice (Fujioka et al., 2007)

Parameter	*0.08% Daidzein*		*0.08% Genistein*	
	Male	*Female*	*Male*	*Female*
Terminal body weight	↓	↔	↓	↔
Lean body mass / fat mass	↔/ ↔	↔ / ↔	↔ / ↔	– / ↓
Food intake	↔	↔	↔	↔
Spleen, kidney, testis or uterus weight	↔	↔	↔	↔
Thymus weight	↓	↓	↓	↓
Liver weight	↔	↔	↔	↓
Whole body BMD / BMC	↑ / ↑	↑ / ↑	↔/ ↔	↔/ ↔
Lumbar spine BMD / BMC	↑ / ↑	↔/ ↔	↔/ ↔	↔/ ↔
Whole femur BMD / BMC	↑ / –	↓ / ↓	↑ / –	↓ / –
Proximal femur BMD / BMC	↔/ –	↓ / –	↔/ –	↓ / –
Middle femur BMD / BMC	↑ / –	↔/ –	↑ / –	↔/ –
Distal femur BMD / BMC	↑ / ↑	↓ / ↓	↑ / –	↔/ –
Periosteal MAR	↔	↔	↔	↔
MS/MB	↑	↔	↑	↔
BFR/BS	↑	↓	↑	↔
Plasma genistein	↔	↔	↑	↑
Plasma daidzein & equol	↑	↑	↔	↔
Plasma testosterone or 17β-estradiol	↔	↔	↔	↔

↑, ↓, ↔ Significantly greater than, lower than or no difference from control value.
– Not reported, presumed to be no significant difference from control value.
From Fujioka et al., 2007 *(737)*.

Authors' conclusion: The effects of isoflavones on bone metabolism during growth depend on sex. Consumption of a diet with 0.08% isoflavones stimulates bone formation in immature male mice and exerts the opposite effect in female mice. These results suggest that endogenous hormonal status influences the efficacy of isoflavone, especially daidzein, on bone metabolism during immaturity in mice.

Strengths/Weaknesses: Strengths of the study include the use of dietary exposure, the animals were administered a specific isoflavone (genistein or daidzein), the diet was defined, and plasma levels of genistein and equol were measured. Multiple endpoints appropriate for bone health were evaluated, and adequate information on the methodologies used to assess bone parameters was provided. A weakness of the study is that timing of exposure was not ideal for assessment of potential effects on early human exposure since the mice were peripubertal at the initiation of treatment.

Utility (Adequacy) for CERHR Evaluation Process: This study has no utility for the evaluation.

Kaludjerovic and Ward *(683)*, supported by the International Life Sciences Institute North America, examined whether neonatal subcutaneous injections of a combination of daidzein and genistein results in greater benefits to bones of the adult male and female mouse than either treatment alone. Outbred CD-1 mice (Charles River) were placed on a control diet (AIN93G) that was devoid of any estrogenic compounds; mice were mated and allowed to deliver **[bedding not described]**. Litters were randomized in to 1 of 5 groups: corn oil (CON, vehicle control), daidzein (DAI, 2 mg/kg bw/day), genistein (GEN, 5 mg/kg bw/day), daidzein plus genistein (DAI+GEN, 2 mg daidzein + 5 mg genistein/kg/day), or diethylstilbestrol (DES, positive control, 2 mg/kg bw/day) **[purity of the substances was not stated]**; daidzein, genistein and diethylstilbestrol were solubilized in dimethyl sulfoxide and suspended in corn oil. Treatments were administered each morning from PND 1 to PND 5 (5 doses) via a single subcutaneous injection. The doses of daidzein and genistein were selected to resemble the quantity and ratio of each isoflavone in soy protein-based infant formula. 1.5 hours after the last dose on PND 5, a subset of pups (n=4-6/group) was killed to verify the serum levels of daidzein, genistein and equol (daidzein metabolite). Body weights were measured weekly. Remaining mice were killed at 4 months of age. Femurs and lumbar vertebrae (LV) were collected and the following endpoints were measured: bone mineral content (BMC) and bone mineral density (BMD) of the left femur and LV1-LV3, biomechanical strength of the femur and LV2, microarchitecture of the femur and LV4. One-way ANOVA was performed to determine the significance of differences in microarchitecture; two-way ANOVA was conducted for all other outcomes with gender and treatment as the main effects. Student-Newman Keuls test was used for comparisons of multiple means when statistical differences were observed.

Body weights were unaffected by treatment. On PND 5, serum isoflavone concentrations of mice treated with genistein and daidzein were higher than all other groups. Females had higher vertebral BMC and BMD than males. All females groups receiving isoflavones or diethylstilbestrol had higher vertebral BMC and BMD than the female control group; Males treated with daidzein or genistein had a higher BMC of LV1-LV3 than males treated with diethylstilbestrol. BMD of LV1-LV3 was lower in diethylstilbestrol-treated males compared to all other groups. Peak load of LV2 was higher among females compared with males; there was a greater gender X treatment effect with daidzein and diethylstilbestrol females having greater vertebral peak load compared to all other groups. Femur BMC was higher among males compared to females and there was an overall treatment effect due to

daidzein, as BMC was higher with daidzein compared with the control and diethylstilbestrol groups. Daidzein treatment resulted in a greater femur BMD compared with all other groups, daidzein males also had higher BMD than diethylstilbestrol-treated males. Females treated with daidzein or diethylstilbestrol and males treated with daidzein or daidzein+genistein had greater femur mid-point peak load than the female control or male diethylstilbestrol groups; all isoflavone groups and diethylstilbestrol had a greater ultimate stiffness at femur midpoint than the control group. Females treated with daidzein, genistein and diethylstilbestrol had higher BV/TV (bone volume/total volume) at the LV4 compared to the control group, and daidzein+genistein had intermediate effects. Treatment with daidzein also resulted in higher BS/BV (bone surface area/bone volume) at the LV4 compared with all other treatment groups. Tb.Th (trabecular thickness) was higher among females treated with genistein and daidzein+genistein compared to all other treatment groups. Treatment with daidzein or diethylstilbestrol resulted in a higher Tb.N (trabecular number) at the LV4 compared to the control group, and daidzein+genistein had intermediate effects. All treatment groups had lower Tb.Sp (trabecular separation) at the LV4 than the control group, and female mice treated with daidzein or diethylstilbestrol had lower Tb.Sp than genistein- or daidzein+genistein-treated mice. At the femur neck, Tb.N was higher among diethylstilbestrol-treated females compared to the other treatment groups; females treated with daidzein and genistein had a higher Tb.Sp compared with the diethylstilbestrol group, and daidzein+genistein had intermediate effects. The outer cortical parameter was higher among females treated with daidzein, genistein, and diethylstilbestrol compared to the control group, and daidzein+genistein had intermediate effects. Treatment with daidzein and genistein resulted in higher cortical area at the femur neck compared with the control group and diethylstilbestrol, and daidzein+genistein had intermediate effects. Bone marrow area was higher among control females or treated with genistein compared with diethylstilbestrol; daidzein alone or with genistein had intermediate effects. At the femur midpoint, females treated with daidzein and genistein alone had a higher BV/TV compared with the control group, and daidzein+genistein had intermediate effects. BS/BV was higher among females treated with diethylstilbestrol compared with daidzein, with all other treatment groups having intermediate effects. Treatment with daidzein resulted in higher Tb.Th, higher cortical thickness, and greater cortical area at the femur midpoint compared with the control group or diethylstilbestrol; genistein alone or in combination with daidzein had intermediate effects. Qualitative assessments revealed that females treated with daidzein or diethylstilbestrol had improved trabecular network at the LV, with genistein and daidzein+genistein having intermediary effects. At the femur neck, females treated with daidzein exhibited visibly greater cortical thickness than all other treatment groups and the female diethylstilbestrol group had visibly improved cortical thickness compared with the control group. At the femur midpoint, the thickness of the cortical wall was greater among females treated with daidzein compared with the female control group.

Authors' conclusion: Neonatal exposure to daidzein and/or genistein had a positive effect on the skeleton of female mice at adulthood, but compared with individual treatments, daidzein+genistein did not have a greater benefit to bone in females or males.

Strengths/Weaknesses: A strength of this study was the use of a control diet (AIN93G) that was devoid of any estrogenic compounds to which genistein and daidzein were added. The doses of daidzein and genistein were selected to resemble the quantity and ratio of each isoflavone in soy protein-based infant formula. It is also useful that a subset of pups (n=4-6/group) were used to verify the serum levels of daidzein, genistein and equol (daidzein metabolite). DES was used as a positive control in

this study. Use of single dose levels for each test compound prevents evaluation of dose-response relationships and thus is a weakness of this report. Other weaknesses were the subcutaneous route of exposure and a relatively short period of exposure compared to humans since the animals were dosed on postnatal days 1 to 5.The primary focus of the study was effects of postnatal exposure to phytoestrogens on bone health and thus reproductive endpoints were not included.

Utility (Adequacy) for CERHR Evaluation Process: This study has limited utility for the evaluation since a subcutaneous route of exposure was used although this is partially compensated by measurement of isoflavones in serum.

3.4.4.3 Non-rodent species

Li et al., 1990 ***(738)***, funding not indicated, examined the effects of soy diet on hypersensitivity in early-weaned pigs. Four sows were fed corn-gluten meal from GD 109 through lactation. Piglets (Hampshire × Yorkshire × Duroc) from those 4 litters were randomly assigned to a soy or milk (control) group (n=16/group). From 7 to 14 days of age, piglets were sensitized by oral gavage with 5 g/day of their respective protein treatment, 48% soybean meal or dried skim milk (control group). Pigs were weaned at 21 days of age and fed a milk-protein or a soybean-meal diet ad libitum until 56 days of age. **[Lactose was listed as the carbohydrate source for the soybean diet but not specified for the milk-protein diet. Lactose was assumed to be the carbohydrate source in the milk-protein diet, but the percentage in diet was not specified. Both diets contained soybean oil.]** One day prior to termination, blood was drawn to obtain lymphocytes and measure IgG titers to soy protein. In addition, dermal soybean hypersensitivity was tested. Half the pigs (2 per litter) were killed at 28 days of age, and the other half were killed at 56 days of age. Duodenum samples were removed to collect lymphocytes and examine villous height and crypt depth by scanning electron and light microscopy. Blastogenic response was determined in lymphocytes. Mitogenic responses were measured in lymphocytes and mononuclear cells. Data were analyzed by ANOVA and Student *t* test.

Body weight gain was reduced at 28 days of age ($P<0.01$). No effects of diet on serum concentrations of zinc, selenium, iron, and copper were detected at 28 and 56 days of age. No significant differences in skin thickness following intradermal injection of soy or milk proteins were observed between the soybean- and milk-diet groups at 27 and 55 days of age. However, IgG titers to soy were significantly increased in both age groups of piglets fed soybean compared to milk-protein diets. At 28 days of age, intestinal villi of pigs fed soybean diets were shorter and broader than those of pigs fed milk-protein diets, but this difference was not observed at 56 days of age. At 28 and 56 days of age, intestinal crypt depth was slightly higher in pigs fed soybean diets. Neither diet was observed to have a significant effect on blastogenic responses of peripheral or intestinal lymphocytes collected at either age and cultured with purified soy proteins, phytohemagglutinin, or pokeweed.

The study authors concluded that pigs sensitized to soybean protein and fed a soybean-meal diet displayed transient hypersensitivity that was evidenced by decreased villous height and increased IgG titers. The effects coincided with reduced growth from 3 to 4 weeks.

Strengths/Weaknesses: A strength of this study is that sows received a corn-corn gluten diet to prevent the passive transmission of soybean-protein antibodies to the offspring. Pigs in each litter were assigned randomly to treatment groups, and it appears that the authors assigned piglets within the same litter to

different treatment groups (i.e., 4 litters were used and at PND 28 necropsy, 8 animals were randomly selected **[2 pigs/litter]** from each treatment for necropsy). Pigs received soybean orally, which represents a relevant route of exposure. Triplicate intestinal villi samples (10 villi/specimen) were measured for each pig. Villous atrophy has been reported in other species after inducing hypersensitivity to soy proteins. A weakness of the study is that the authors used only one dose level of soy, so dose-response relationships could not be evaluated. Pigs originated from only 4 litters. There was no indication that the authors controlled for litter effect during their data analyses. Aside from soybean meal, there were other differences between the two weanling diets. Abnormal villous morphology and increased serum IgG titers corresponded with a period of decreased growth in soy-fed weanlings. As the authors point out, "low pre-weaning feed intake, which commonly occurs when weaning at 3 wk or less, results in a transient hypersensitivity to dietary antigens." It is interesting that skin-fold thickness was doubled in pigs maintained on a milk protein-based diet and given a milk-protein injection on PND 27, particularly given that milk-protein allergies are common in children. There was no increase in serum anti-soy antibody IgG titers in milk protein-fed pigs, but anti-milk-protein IgG was not tested. The contribution of early weaning stress to these effects is not known.

Utility (Adequacy) for CERHR Evaluation Process: This report is of limited utility in the evaluation process.

Zijlstra et al., 1996 ***(739)*****,** funding not indicated, examined the short-term metabolic effects in neonatal piglets fed soy formula. Two separate experiments were conducted 12 months apart. In the first experiment, a catheter was inserted into the umbilical artery of 1-day-old neonatal pigs. Pigs were fed by gavage dosing with casein-whey protein formula or hydrolyzed-soy formula for 36 hours and then received only water for 12 hours. A catheter was inserted into the portal vein of neonatal piglets (large White × Pig Improvement Company), and baseline blood samples were drawn from the catheters 1 hour later. The piglets were gavaged with formula (20 mL/kg bw) containing cow-milk protein (40:60 casein:whey ratio), hydrolyzed-soy protein, or intact-soy protein. Arterial and portal blood samples were collected at 15, 30, 60, and 120 minutes following dosing. The second experiment was similar to the first except that only an arterial catheter was inserted, and blood sampling was extended to 3 hours. There was also a difference in formula carbohydrate source. In experiment 1, carbohydrate sources were glucose polymers in soy-protein formulas and lactose in cow-milk protein formula. In experiment 2, the soy and cow-milk formulas were standardized to contain equal amounts of reduced lactose and glucose polymers. Soy diets were fortified with *d,l*-methionine and *l*-lysine in both experiments. Parameters measured included plasma amino acid levels using anion-exchange chromatography, insulin and glucagon levels by RIA, and glucose concentrations using a glucose-oxidase method. In experiment 2, piglets were killed following the last sampling time and gastrointestinal contents were weighed to determine gastric emptying. Each experiment was conducted as 4 replicates of 6 littermates. Within each replicate, 2 piglets were randomly assigned to each of the 3 treatment groups. Therefore, there were a total of 8 piglets in each treatment group in experiments 1 and 2. **[Data were analyzed according to a randomized complete block design, but the statistical tests were not specified.]**

In the 48-hour period prior to formula dosing and blood sampling in both experiments, piglets fed casein-whey formula gained weight, while piglets fed hydrolyzed-soy formula lost weight. Diarrhea was observed in 15 of 16 piglets fed the hydrolyzed-soy formula in experiment 1 and in all piglets

fed the casein-whey and hydrolyzed-soy formulas in experiment 2. The study authors noted that concentrations of glucoamylase and maltase are low in the digestive tracts of piglets. Therefore, there may have been impaired digestion and absorption of the carbohydrates used in soy formulas in experiment 1 and all formulas in experiment 2. The authors also cited studies noting that soy proteins were shown to cause small intestine mucosa damage resulting in malabsorption and diarrhea in pigs.

In experiment 1, differences between amino acid concentrations in portal compared to arterial blood following dosing with all formulas indicated absorption from the gut. In piglets fed casein-whey formula, the difference between amino acids in portal compared to arterial blood peaked within 15 minutes, indicating rapid digestion and absorption of proteins. The post-feeding rise in amino acid levels in portal blood was slower and peaked at a lower level with soy formulas, suggesting attenuated digestion and absorption of proteins. Higher concentrations of amino acids in portal compared to arterial blood were maintained for 2 hours following feeding only in the piglets given hydrolyzed soy. In experiment 2, patterns of amino acid concentrations in arterial blood were similar to those in experiment 1. Weight of material remaining in stomach and small intestine did not differ between groups, leading study authors to conclude that differences in gastric-emptying rates were not the reason for altered plasma concentrations between the different feeding groups. In experiment 1, insulin and glucose concentrations were significantly increased at 15–30 minutes after feeding in arterial and portal blood of piglets fed casein-whey compared to either soy formula. No significant effects on glucagon levels were detected. In contrast to experiment 1, no significant differences were detected in arterial blood concentrations of insulin, glucagon, or glucose at 15–30 minutes following feeding in experiment 2.

The authors concluded that "hydrolyzed soy protein did not result in elevated nutrient concentrations or hormone responses compared with intact soy or **[cow]** milk protein." Standardization of carbohydrate sources among formulas in experiment 2 but not experiment 1 was suggested by study authors as a possible reason for differences in results between the first and second experiments.

Strengths/Weaknesses: A strength is that during the experiments, formulas were stirred constantly to ensure homogeneity, and formulas were replaced every 12 hours. The composition of the various formulas was provided although there were no analyses of the isoflavone content. Soy hydrolysis was examined using HPLC. The authors partially controlled for litter effect by using 2 piglets/litter for each treatment group. Treatment groups were equally represented in each replicate. Pigs received formulas orally, which represents a relevant route of exposure and allowed control of the dose administered. To avoid potential effects on small intestine structure and function prior to sample collection, pigs were maintained on the hydrolyzed soy-protein formula prior to the metabolic study when both hydrolyzed- and intact-soy protein were used. The authors monitored the amount of dry matter in the stomach and 3 segments of the small intestine to confirm that differences in absorption were not related to differences in transit time through these gastrointestinal segments. Statistical analyses included a randomized complete-block design to account for different study replicates, although it is not clear that the authors considered the litter in their analysis. A weakness is that the authors used only 1 dose level of hydrolyzed- and intact-soy protein, so dose-response relationships could not be evaluated. In both experiments, hydrolyzed soy protein-treated pigs lost weight, whereas control pigs fed casein-whey protein gained weight over the monitoring period prior to formula dosing. In the first experiment, hydrolyzed-soy protein-fed pigs had diarrhea, and both

hydrolyzed soy protein- and casein whey-fed groups had diarrhea in experiment 2. Pigs fed casein-whey protein had greater glucose and insulin concentrations in experiment 1, which the authors corrected in experiment 2 by normalizing dietary carbohydrate composition. Each of these factors may have contributed to differences in response. The authors report pooled SEM, so it is not possible to determine the variability around individual means.

Utility (Adequacy) for CERHR Evaluation Process: This report is of no utility in the CERHR evaluation process.

3.5 Utility of Data

Studies contained in Chapter 3 were categorized by the Expert Panel as being of "no," "limited," or "high" utility. Only those studies of "limited" or "high" utility are presented in **Table 153** to **Table 157** and considered in preparing summary conclusions on the developmental toxicity of soy formula.

3.5.1 Human Data

There were no data identified for humans on developmental exposures to the individual isoflavones found in soy formula.

In contrast, there are a large number of papers describing growth or other health parameters in humans exposed to soy infant formula. Twenty-seven were judged to have limited utility in the evaluation process (**Table 153**). Commonly encountered limitations included the non-random or unspecified method of assignment to feeding groups, the use of self-selected breast- and formula-feeding mothers, failure to control for the reasons for which soy formula was used, and the early and inconsistent introduction of solid foods. Studies that compared outcomes in children randomized to soy or cow-milk formula were considered the most reliable, particularly when parents and outcome assessors were masked to formula assignment. The studies also were evaluated based upon adequate sample size by gender and feeding group, presence or absence of longitudinal follow-up, validation of exposure to soy formula, and appropriate adjustment for potential confounding variables.

The two most recent studies addressing potential health effects among infants fed soy formula, Bernbaum *et al.*, 2008 *(574)* and Gilchrist *et al.*, 2009 *(578)*, demonstrate the inherent difficulties in the execution and evaluation of human epidemiological studies. These studies, although deemed to have no utility for this report, have resulted in the initiation of a larger study utlizing the results of the pilot Bernbaum *et al.*, 2008 *(574)*, and the continuation of data collection from the interim analyses reported in Gilchrist *et al.*, 2009 *(578)*. Clarification with regard to how these studies may provide insight to the question of interest in this report seems appropriate.

- Researchers at the NIEHS, CDC, and the FDA have collaborated with 2 academic centers to conduct the Study of Estrogen Activity and Development (SEAD), a series of mostly cross-sectional pilot studies designed to establish methods for future larger studies evaluating the estrogenic effects of soy infant formulas (or any putative estrogenic exposure) on the developing infant *(740)*. SEAD included equal numbers of infants fed soy formula, cow milk formula, or breast milk. Findings from the study evaluating breast and genital development in infants, i.e., breast buds, breast adipose tissue, testicular volume and position, vaginal discharge and cell maturation were published in Berbaum *et al.*, 2008 *(574)*. **[The Expert Panel reviewed the Bernbaum et al. (2008) study and considered it of "no utility" for the**

current evaluation.] The sample size was very small (once gender and age were considered) and the Expert Panel determined the study lacked statistical power to detect any relevant associations. The proposed mixed cross-sectional study design called for two boys and 2 girls in each of 7 age intervals (with a range of < 48 hours through 6 months of age) across three pre-specified feeding regimens (with up to 4 visits per child allowed). Although up to 4 visits per child were permitted, very few longitudinal measurements were obtained and those that were captured demonstrated the potential benefits of time course data in this evaluation given the patient by age variability observed for genital development. Given the variability observed and insufficient data to adequately estimate effect sizes, this study was categorized as no utility.

- The Arkansas Children's Nutrition Center (ACNC) is currently conducting a prospective, longitudinal study in children aged 2-3 months through 6 years who were breast-fed, cow milk formula-fed, or soy formula-fed as infants. Assessments of growth, development, body composition, endocrine status, metabolism, organ development, brain development, cognitive function, language acquisition, and psychological development will be obtained at 3, 6, 9, 12, 18 months and 2, 3, 4, 5, and 6 years. An interim examination of the data summarized differences in hormone-sensitive organ size at 4 months in infants who were fed soy formula (SF) (n=39, 19 males and 20 females), milk formula (MF) (n=41, 18 males and 23 females), or breast milk (BF) (n=40, 20 males and 20 females) *(578)*. All BF infants were stated to be exclusively fed breast milk the entire study time. Fifty-four percent of the MF infants were stated to be exclusively fed MF from birth, 41% switched from BF to MF within 4 weeks, and 5% switched between 4 and 8 weeks. 23% of SF infants were exclusively fed SF from birth, 45% were switched to exclusive SF feeding within 4 weeks, and 32% were switched to SF between 4 and 8 weeks. At 4 months, anthropometric measures (weight, length, and head circumference) were assessed using standardized methods, and body composition was assessed by air displacement phlethymography. Breast buds, uterus, ovaries, prostate and testicular volumes were measured by ultrasonography. The benefit of longitudinal data in characterizing differences in developmental endpoints across the exposure groups is a valuable study design feature. However, when exposure is mixed due to transitions in diet, the effects may be attenuated or exaggerated which makes the results thus far of no utility. However, if continued recruitment into the exposure groups increases the sample sizes whereby diet transitions are not permitted or data are collected prior to these transitions then the study would have greater value.

Experimental Animal Data on the Individual Isoflavones Found In Soy Formula

The interpretation of many animal studies was hampered by the use of single dose levels, particularly when those dose levels were well above levels relevant to humans, use of treatment time periods that extended beyond development, the lack of reporting of litter data, and the lack of litter-based analysis. Route of exposure was a potentially important issue in the interpretation of studies. A working group of the expert panel met on June 8-9, 2009 to consider the impact of factors such as these in developing a consistent strategy for preparing strengths/weaknesses and utility statements for the experimental animal studies described in Chapter 3 of the Expert Panel report on Soy Infant Formula. This initial strategy was refined by the expert panel during its December 16-18, 2009 meeting, resulting in the development of the following guidelines used to evaluate individual studies.

1. **Lifestage of Exposure**
   Most weight in the assessment was given to those studies that exposed developing animals to soy, or in most cases the specific isoflavone genistein, solely during the period from birth to weaning because this window of exposure best approximates the timing of soy formula ingestion in infants.

2. **Route of administration**
   The oral route of dosing is considered most relevant for humans because exposure to the isoflavones found in soy formula occurs through the diet. The expert panel tried to preferentially evaluate studies that used oral administration since it best mimics the human route of exposure. However sc injection studies, particularly those using doses which produce blood levels representative of those measured following human exposure were also considered.

3. **Control for litter effects**
   Failure to use litter as the experimental unit in data analysis when this is appropriate is an important design weakness for gestational exposure studies, but did not necessarily result in a study being considered of "no" utility.

4. **Sample size**
   No pre-determined number was set as a cut-off for acceptable sample size. This decision will depend on the endpoint and animal model being assessed. For example, studies using sophisticated techniques, addressing specific experimental questions, or those that use primates may be justified in use of relatively small sample size.

5. **Single exposure level studies**
   Use of a single exposure level is not necessarily a study weakness and depends on the rationale for not utilizing multiple exposure levels.

6. **Use of positive control**
   Lack of a positive control is not counted against a study, but a negative study with a "failed" positive control for expected specific effects, e.g., estrogenic, will be considered of "no utility."

7. **Number of endpoints assessed**
   It is not necessarily a study weakness if only one or a small number of endpoints are assessed. This could reflect investigator's interest in a specific tissue and/or mechanism of action.

Developmental toxicity studies of individual isoflavones found in soy formula were conducted in rats, mice, and pigs exposed through diet and by sc injection (**Table 154** to **Table 156**). Single agent studies primarily focused on genistein with very little data for other isoflavones such as daidzein and equol. In infants, the primary isoflavone exposure is from genistein and daidzein although equol has been detected in the plasma and urine of some infants *(84; 94)*. In general, the most informative data were available from oral exposure studies in rats and sc exposure studies in mice. Prenatal endpoints such as offspring growth and survival were reported for rats exposed through diet *(633; 637; 667)*. One study in rats examined genistein for possible teratogenicity *(612)*. General postnatal endpoints such as growth, survival, and developmental milestones were examined in offspring of rats dosed through diet *(239; 633; 637)* and in pups gavaged during the neonatal period *(625)*. Endocrine-mediated endpoints such as age at puberty, estrous cyclicity, spermatogenesis, and/or histopathology of male and female reproductive organs were examined in studies in which mice were exposed to genistein by sc injection or orally in prenatal and/or postnatal periods [334; 591; 592; 602-604; 645] and rats were exposed orally or by sc injection during gestation, lactation, and/or postweaning *(239; 243; 625; 633; 634; 637; 640; 642)*. Effects on mammary development and susceptibility to chemically induced carcinogenesis were examined in mice and rats exposed orally or parenterally during prenatal or postnatal development *(209; 323; 644; 645; 654; 655)*. Development of sexually dimorphic regions of the brain and sexually dimorphic behaviors were assessed in rats exposed orally or parenterally during prenatal and/or postnatal development [243; 665-667; 669; 674; 720]. A limited number of studies addressed the effects of genistein exposure during development on the thyroid *(242)* and the immune system of rats *(680; 682; 684)*. A common limitation of many studies was that exposures occurred during development and through adulthood, thus complicating the interpretation of the data.

Experimental animal studies on developmental effects of soy formula or other soy foods have been performed in rats, mice, pigs and non-human primates. Almost 30 experimental animal studies were found to have "limited" or "high" utility for the evaluation process (**Table 157**). Of these studies, 1 small study in rats *(622)* and 2 studies in marmosets *(708; 709)* involved administration of soy milk or infant formula, and only the marmoset studies involved direct administration of soy formula to infant animals. A study conducted in pigs *(738)* used a 48% soybean meal-based liquid diet for neonatal animals. This soybean-meal content is higher than the 14.6–16% content of soy-protein isolate in marketed formulas, see **Section 1.1.3**. Most of the developmental studies performed in rodents examined the effects of dietary soy products or of soy-isoflavone preparations added to soy-free diets, and it is not clear to what extent the effects of soybean meal are similar to those of commercial isolates. The dietary interventions used in the experimental animal studies differ from one another and it is not clear which, if any, are appropriate models for soy infant formula. Dose-response information is available only from the study of McVey *et al.*, 2004 *(697; 698)*, in which an isoflavone mixture was added to a soy-free feed giving rise to 5 different isoflavone-intake levels. The use of this study in the assessment of possible toxicity of soy formula assumes that the isoflavone content of soy formula is the toxicologically important component.

3.6 Summary of Developmental Toxicity Data

3.6.1 Human Data

No human data were identified on exposures to the individual isoflavones found in soy formula.

Studies on outcomes after feeding soy formula in infancy are summarized in **Table 153**. Most of

Table 153. Studies of Human Infants Exposed to Soy Formula

Comparison Groups	Major findings	Reference	Utility
Growth and Nutrition			
Infants with immunoglobulin E-mediated cow's milk aller-gy fed breastmilk (n=32) (reference) and infants randomly assigned to one of three special formulas dur-ing a feeding period of 6–12 months of age: • soy formula (n=32), • a casein hydrolysate (n=31) • a rice hydrolysate (n=30)	The 6–12 month change in weight-for-age *z*-score for the soy formula group was significantly lower compared to the rice (0.18, $P=0.019$) and casein (0.16, $P=0.029$) groups. This indicates better short-term weight gain with hydrolyzed products when compared with soy formula.	Agostoni et al., 2007 *(541)*	Limited
Infants randomized to 1 of 2 different soy formulas (n=10/group) and 10 breast-fed infants (for comparisons to 4 months of age).	Soy formula-fed infants at 4 months of age: • ↓bone mineral density • ↑ plasma zinc Unable to detect difference between soy formula-fed infants and his-torical controls in these parameters at 6 and 12 months. No effect on measures of growth and serum chemistries.	Chan et al., 1987 *(510)*	Limited
Infants fed soy infant formula (n=28) and infants fed cow-milk formula (n=30) for 6 months. The soy formula was not methionine-supplemented as is modern soy formula.	Soy-fed infants, especially girls: • lagged in volume consumed from 2 to 4 months of age • lagged in growth from 2 to 4 months of age • ↓weight gain per fluid ounce of formula • ↓vomiting • ↓number of infants hungry after feeding • ↑ loose stools No effect on anemia or hematologic parameters.	Cherry et al., 1968 *(511)*	Limited
Infants followed for 1 year on: • soy formula (n=11) • cow-milk formula (n=11) • human milk (n=9)	Unable to detect a difference in bone width or bone mineral content. Differences in serum and urine minerals and bone-related hormones were consistent with homeostatic adjustments to different mineral con-tent in the food sources.	Hillman et al., 1988a, 1988b *(516; 517)*	Limited
Infants on soy formula (n=20) and infants on cow-milk formula (n=20) for 16 weeks.	Unable to detect a difference between the 2 groups in: • growth parameters • hematology measures • blood chemistry	Jung and Carr, 1977 *(518)*	Limited
Infants selected at 6 weeks of age and followed for 1 year: • infants on soy formula (n=13) • infants on cow-milk formula (n=20) • breast-fed infants (n=26)	↑ weight at birth Unable to detect growth differences between groups after 6 weeks of age. Soy-fed infants showed slower bone mineralization at 3 months but not thereafter.	Köhler et al., 1984 *(520)*	Limited

Table 153 (continued)

Comparison groups	Major findings	Reference	Utility
Very low birth-weight infants given soy formula, cow-milk formula, or high-calorie cow-milk formula until 3–4 months of age.	After three weeks of age: • ↓serum phosphorus • ↑serum alkaline phosphatase	Kulkarni et al., 1984 *(522)*	Limited
Infants fed soy formula (n=18) or cow-milk formula (n=17) for 12 months.	↓bone mineral content Unable to detect differences in energy intake or growth.	Steichen and Tsang, 1987 *(530)*	Limited
Infants on soy formula with (n=73) or without (n=73) supplemental nucleotides, and infants breast-fed for 2 months and then given cow-milk formula (n=67). Infants followed to 12 months of age.	Groups were not comparable in weight and length at birth. No group differences in growth were detected after 6 months of age.	Lasekan et al., 1999 *(523)*	Limited
Infants given 1 of 2 different soy formulas (n=21/group), infants given cow-milk formula (n=20), and breast-fed infants (n=10), followed for 1 year. Solid food introduced at 3 months in breast-fed and 4 months in formula-fed groups.	↑length gain and ↑serum 1,25-dihydroxyvitamin D in 1 of the soy-formula groups at 8 weeks and thereafter. ↑serum phosphorus in combined formula groups at 8 weeks. Unable to detect effect of feeding type on: • weight and head circumference • bone mineral content and bone width • blood chemistry	Mimouni et al., 1993 *(524)*	Limited
Infants being breastfed (n=401) and infants assigned to either cow-milk (n=839) or soy based formula (n=239) based on family history of allergy. Evaluated monthly until 6 months of age and bi-monthly thereafter.	No differences in length or weight were detected between the 3 feeding groups.	Sellars et al., 1971 *(526)*	Limited
Infants exclusively fed soy formula (n=20), cow-milk formula (n=19), or breast-fed (n=17) for 4 months and followed until 6 months of age.	↑bone mineral content and bone width in soy formula-fed infants compared to breast-fed infants. Unable to detect group differences in growth and serum measurements related to bone accretion.	Venkataraman et al., 1992 *(532)*	Limited
Allergy, Immunology, and Gastrointestinal Effects			
Infants with a family history of major allergy assigned to soy formula (n=79) or cow-milk formula (n=201), followed 17 years; 48 infants with a family history of major allergy were breast-fed.	Breast fed children were significantly less likely to develop allergy after 3 years than soy or cow-milk formula fed children. Unable to detect a difference in development of allergic disease by formula type.	Gruskay, 1982 *(550)*	Limited
Infants with cow milk allergy diagnosed at age 2–11 months were randomly assigned to extensively hydrolyzed formula (n=90) or soy formula (n=80) and followed until 2 years of age.	Parents suspected adverse reaction in 28% of subjects on soy formula and 11% of subjects on extensively hydrolyzed formula. Among children younger than 6 months, 40% were suspected by parents of having adverse reaction to soy formula.	Klemola et al., 2002 *(554)*	Limited

Table 153 (continued)

Comparison groups	*Major findings*	*Reference*	*Utility*
Infants with milk allergy fed whey formula (n=76) or soy formula (n=72), examined at age 2, 3, and 4 years.	Non-significant increase in soy IgE levels in soy formula-fed infants. *(OR 2.28, 95% CI 0.90–5.76; P=0.082)* No effect on prevalence of peanut IgE positivity: *(OR 1.27, 95% CI 0.35–4.71; P=0.717)*	Klemola et al., 2005 *(555)*	Limited
Children with peanut allergy (n=49), children with atopy (n=70), and non-allergic children (n=140) surveyed for infant feeding history.	Soy consumption was a significant and independent risk factor for peanut allergy (*Adjusted OR 2.61; 95% CI 1.31–5.20).*	Lack et al., 2003 *(558)*	Limited
Infants given 1 of 2 different soy formulas (n=92, 94), one of which contained added nucleotides, and breast-fed children who were weaned to cow-milk formula at 2 months (n=81); 1-year follow-up.	Unable to detect a difference by feeding group in: • antibody response to immunizations (except *Hemophilus influenza B*) • parent-reported diarrhea • otitis media No consistent differences were detected between soy and cow-milk formula-fed infants. in: • immune status, • maturation • level of immunocompetence	Ostrom et al., 2002 *(548)* Cordle et al., 2002 *(547)*	Limited
Infants with cow's milk allergy fed soy formula (n=84, mean starting age=7.8 months) or a extensively hydrolyzed whey formula (HWF) (n=84, mean starting age=7.5 months), examined until 48 months of age.	• ↑percentage of energy • ↑intake of zinc and vitamin E • ↓riboflavin No effect on: • percentages of abnormally low lab values • percentages of high alkaline phosphatase • growth measured between 1 to 4 years • nutritional status	Seppo et al., 2005 *(527)*	Limited
Thyroid Function			
Infant with congenital hypothyroidism who was fed soy formula (n=1).	Oral thyroxine therapy did not decrease thyroid-stimulating hormone until cow-milk formula was substituted for soy formula.	Chorazy et al., 1995 *(570)*	Limited
Infants fed soy formula (n=8) and infants fed non-soy formula (n=70).	Infants with congenital hypothyroidism fed soy formula had prolonged increase of TSH levels compared to infants with congenital hypothyroidism fed non-soy formula. No effect on: • total T4 between groups • weight and height • weight for height	Conrad et al., 2004 *(571)*	Limited

Table 153 (continued)

Comparison groups	Major findings	Reference	Utility
Infants with congenital hypothyroidism who were fed soy formula (n=3).	Switching from soy formula to cow-milk formula resulted in increased absorption of thyroid replacement doses.	Jabbar et al., 1997 *(572)*	Limited
Children with goiter related to soy formula-feeding (n=3).	Improvement with discontinuation of soy formula (n=2) or addition of iodine (n=1).	Shepard et al., 1960 *(573)*	Limited
Reproductive Function or Tissue Effects			
Adults with breast cancer (n=372) and without breast cancer (n=356) who had been fed soy formula, cow-milk formula, or breastmilk	A reduced, but non-significant, association was found between soy formula intake and breast cancer. Soy formula only during fist 4 months of life: *OR 0.42, 95% CI 0.13–1.40.* Soy formula only during 5–12 months of age: *OR 0.59, 95% CI 0.18–1.90*	Boucher et al., 2008 *(575)*	Limited
Girls with premature thelarche (n=130) and age-matched control subjects. Retrospective questioning of parents about infant feeding.	Unable to detect a significant association overall between premature thelarche and soy infant formula intake. Restriction of multivariate analysis to subjects with thelarche before age 2 years showed significant association: *OR 2.7; 95% CI 1.1–6.8, P=0.029*	Freni-Titulaer et al., 1986 *(576)*	Limited
Adults who had been fed soy (n=248) or cow-milk formula (n=563) during infancy as part of a controlled trial. Interviewed at 20–34 years of age	Unable to detect infant feeding-related differences in adult height, weight, body-mass index, or sexual maturation history. Duration of menstrual bleeding was 0.37 days longer and severe menstrual discomfort was more cmmon in women fed with soy formula than with cow-milk formula.	Strom et al., 2001 *(32)*	Limited
Other Endpoints			
Infants : • on soy formula (n=16, some with cholesterol added) • on cow-milk formula (n=10) • breast-fed with supplemental cow-milk formula (n=12)	Breast-fed infants had the highest serum cholesterol levels and lowest fractional cholesterol synthesis rate. There was a significant inverse relationship between cholesterol intake and fractional cholesterol synthesis.	Cruz et al., 1994 *(199)*	Limited

Table 154. Experimental Studies with Developmental Toxicity Endpoints in Mice Exposed to Genistein, Daidzein, or Equol

Animal Model and Study Design	Endpoints	NOEL	LOEL	Reference	Utility
Growth, Reproductive System and Endocrine-Related Endpoints					
CD-1 mice *Genistein:* 0, 6.25, 12.5, 25 or 37.5 mg/kg bw/day (expressed as genistein equivalent dose) Oral dosing from pipette PND 1–5	• ↑multi-oocyte follicles • ↓percent of plug positive dams delivering live pups (based on combining all ages that were assessed)	6.25	12.5	Jefferson et al., 2009 *(217)* Oral portion	High (Oral)
	• ↑uterine weight • ↑number of abnormal estrous cycles • ↑uterine weight	12.5	25		
	• ↓total number of live pups per litter (based on combining all ages that were assessed) • ↓average number of live pups per litter • delay in age at vaginal opening • ↓number of litters with live pups/dam (based on combining all ages that were assessed)	25	37.5		
CD-1 mice *Genistein:* 0, 25, 37.5, or 75 mg/kg bw/day Oral dosing from pipette PND 1–5	• ↑uterine weight	37.5	75	Jefferson et al., 2009 *(217)* Oral portion	High (Oral)
CD-1 mice *Genistein:* 0.17 mg/kg bw/day Oral gavage GD13 through GD17	• ↑frequency of hypospadias in males (25%)	–	0.17 (Single dose)	Vilela et al., 2007 *(587)*	Limited (Oral)
CD-1 mice *Genistein:* 0 or 50 mg/kg bw/day SC injection on PND 1–5	• ↓implantation sites (both number of mice and number of sites/mouse) • ↓percent pregnant mice • ↓number of corpora lutea	–	50 (Single dose)	Jefferson et al., 2005 *(592)*	Limited (Parenteral)
CD-1 mice *Genistein:* 0, 0.5, or 10 mg/kg bw/day SC injection on GD 15–18	• ↑body weight gain • onset of vaginal opening, 0.5–1 day earlier • ↑estrous cycle length, 1.2–2 days • ↑length of diestrus	–	0.5	Nikaido et al., 2004 *(585)*	Limited (Parenteral)
	• ↓number of animals with corpora lutea at 4 weeks of age, transient effect	0.5	10		

Table 154 (continued)

Animal Model and Study Design	*Endpoints*	*NOEL*	*LOEL*	*Reference*	*Utility*
$B6D2F_1$ mice *Genistein:* 0, 0.1, 0.5, 2.5, or 10 mg/kg bw/day Gavage on GD 12 through PND 20	• ↓ fragmented eggs on PND 105	–	0.1[a]	Fielden et al., 2003 *(604)*	Limited (Oral)
	• ↓ percent of 1-cell fertilized eggs on PND 315	0.1	0.5		
	• ↓ anogenital distance in males on PND 21 • ↑ percent of in vitro fertilization by sperm on PND 105 and 315 • ↑ fragmented eggs on PND 315	2.5	10		
	No effect on: • number of dams giving birth to live pups • pup survival to PND 4 or 21 • litter size • pup or litter weight • sex ratio of pups • sperm count or motility • seminal vesicle, testis • adult body weight • testicular gene expression of estrogen and androgen receptors.	10 (High dose; no effect)	–		
CD-1 mice *Genistein:* 0 or 50 mg/kg bw/day SC injection on PND 1–5	• ↓ number of embryos collected per mouse following HCG administration • ↓ number and size of implantation sites in mice who were recipients of transferred blastocysts obtained from untreated mice	–	50 (Single dose)	Jefferson et al., 2009 *(594)*	Limited (Parenteral)
	No effect on: • number of ovulated eggs • timing of development or percentage of embryos to reach blastocyst age • number of live pups, or litter size	50 (High dose; no effect)	–		
CD-1 mice *Genistein:* 0 or 50 mg/kg bw/day SC injection on PND 1–5	• ↑ number of reproductive lesions • no corpora lutea • abnormal oviduct histology • uterine squamous metaplasia • cystic endometrial hyperplasia • uterine carcinoma	–	50 (Single dose)	Newbold et al., 2001 *(334)*	Limited (Parenteral)

Table 154 (continued)

Animal Model and Study Design	*Endpoints*	*NOEL*	*LOEL*	*Reference*	*Utility*
CD-1 mice *Genistein:* 0, 0.5, 5, or 50 mg/kg bw/day SC injection on PND 1–5	Distribution of females in various stages of estrous cycle at 2 months of age	–	0.5	Jefferson et al., 2005 *(592)*	High (Parenteral)
	↓number of dams with litters at 2 and 6 months of age **[Author's report that "a trend test showed a statistically significant decrease in the number of mice with litters at 2 and 6 months of age with increasing dose as determined by the Cochran-Armitage test ($P < 0.05$)."]**	–	≥0.5 (Significant negative trend)		
	• ↓number of live pups born to dams of 2, 4 and 6 months of age (when all ages looked at simultaneously)	0.5	5		
	• ↓corpora lutea per dam at 4 months of age	5	50		
	No effect on: • number of plug positive mice • number of ovulated oocytes following treatment of mice with human chorionic gonadotropin at 4 months of age • overall treatment effects on serum progesterone, 17β-estradiol, or testosterone levels	50 (High dose; no effect)	–		
ICR mice *Genistein:* 0 or 1000 mg/kg bw/day Injection on PND 1–5	• ↓testicular mRNA expression of ERα, ERβ, AR and laminin-γ2	–	1000 (Single dose)	Adachi et al., 2004 *(601)*	Limited (Parenteral)
	No effect at 12 weeks of age on: • body weight • absolute or relative testis weight • histological changes in the testes	1000 (High dose; no effect)	–		
CD-1 pregnant mice *Genistein:* 0, 0.2 or 2 mg/kg bw/day IP injection from GD 1–21	No effect on: • gross or histological anomalies of the uterus • *Hoxa 10* mRNA expression in the uterus	2 (High dose; no effect)	–	Akbas et al., 2007 *(336)*	Limited (Parenteral)
CD-1 pregnant mice *Daidzein:* 0 or 2 mg/kg bw/day IP injection from GD 1–21					Limited (Parenteral)

Table 154 (Continued)

Animal Model and Study Design	*Endpoints*	*NOEL*	*LOEL*	*Reference*	*Utility*
CD-1 mice *Genistein:* 1, 10, or 100 µg/day (0, 0.5, 5, or 50 mg/kg bw/day) SC injection on PND 1–5	• ↑ multi-oocyte follicles	5	50	Jefferson et al., 2002 *(591)*	Limited (Parenteral)
C57BL/6, ERα and ERβ mice *Genistein:* 0, 1, 10, or 100 µg/day (0, 0.5, 5, or 50 mg/kg bw/day) SC injection on PND 1–5	• ↑ multi-oocyte follicles in erα • no effect in ERβ	0.5	5		
CD-1 mice *Genistein:* 50 mg/kg bw/day SC injection from PND 1–5	• ↑ number of multi-oocyte follicles • fewer single oocytes	–	50 (Single dose)	Jefferson et al., 2006 *(593)*	Limited (Parenteral)
CD-1 mice *Genistein:* 0 or 10 mg/kg bw/day SC injection on PND 15–18	• acceleration of vaginal opening	–	10 (Single dose)	Nikaido et al., 2005 *(595)*	Limited (Parenteral)
	No effect on: • body weight • estrous cycling • polyovular ovarian follicles • morphological abnormalities in vaginal uterine epithelium • mammary gland development	10 (High dose; no effect)	–		
ICR mice *Genistein:* 0, 7, 71, and 714 mg/kg bw/day SC injection on PND1–5	• ↓ AR mRNA expression in testis	7	71	Shibayama et al., 2001 *(602)*	Limited (Parenteral)
	• ↓ ERα mRNA expression in testis	71	714		
	No effect at 12 weeks of age on: • testis weight • sperm count • sperm motility	714 (High dose; no effect)	–		

Table 154 (continued)

Animal Model and Study Design	*Endpoints*	*NOEL*	*LOEL*	*Reference*	*Utility*
Male Han-NMRI mice *Genistein:* 0, 0.1 or 1 mg/day (50 or 500 mg/kg bw/day) SC injection on PND1–3	↓ventral prostate weight in adulthood (histological changes observed)	–	50	Strauss et al., 1998 *(603)*	Limited (Parenteral)
	• ↓ coagulating gland weight • ↑ histologic abnormalities (hyperplasia and disorganization of the epithelium of the prostatic collecting ducts, ventral lobes, and seminal vesicles) • ↑ fibromuscular stroma and inflammatory cells in posterior periurethral region	50	500		
	No effect on *c-fos* mRNA expression in prostatic urethra	500 (High dose; no effect)	–		
Mammary Gland Development and Carcinogenesis					
CD-1 mice *Genistein:* 0 or ~0.7–0.8 mg/kg bw/day Injection on GD 15–20	• ↑ mammary gland epithelial area on PND 35 (but not on PND 25 or PND 46) • ↑ density of tebs on PND 35 and 45 • ↑ PND 25 body weight • delayed eye opening • accelerated timing of vaginal opening	–	~0.7–0.8 (Single dose)	Hilakivi-Clarke et al., 1998 *(646)*	Limited (Parenteral)
	No effect on: • number of offspring born • PND 1 body weight • differentiation of breast tissue (assessed using density of tebs and lobuloalveolar units) • serum 17β-estradiol • estrous cyclicity	~0.7–0.8 (High dose; no effect)	–		
Sprague Dawley rat *Genistein:* 50 µg/day (authors estimated the doses received were 1.25–3.3 mg/kg bw) SC injection on PND 8–20	• ↓ Mammary epithelial density • ↓ Terminal end bud numbers • ↑ Increased lobuloalveolar structures at 8 weeks • ↑ BRCA1 expression • ↑ ERα expression in lobules at 3 and 8 weeks	–	50 (Single dose)	Cabanes et al., 2004 *(648)*	Limited (Parenteral)

Table 154 (continued)

Animal Model and Study Design	*Endpoints*	*NOEL*	*LOEL*	*Reference*	*Utility*
CD-1 mice *Genistein:* 0, 0.5, 5, or 50 mg/kg bw/day SC injection on PND 1–5	• mammary gland hormone receptor levels • ↑ PR protein expression at 5 weeks • ↓ ERα mRNA at 5 and 6 weeks (combined)	–	0.5	Padilla-Banks et al., 2006 *(647)*	Limited (Parenteral)
	• ↑ ductal elongation at 6 weeks • ↑ ERβ mRNA in mammary gland at 5 and 6 weeks (combined)[a]	–	0.5[a]		
	• ↓ number of tebs at 6 weeks • ↓ number of branch points at 5 but not at 6 weeks Mammary gland morphology at 9 months: • ↓ alveolar development • dilated and/or dilated beaded ducts	0.5	5		
	• ↓ number of tebs at 5 weeks • ↓ mammary gland ductal growth at 5 or 6 weeks • ↓ average area of mammary gland at 5 or 6 weeks • altered estrous cyclicity	5	50		
	• ↓ number of pups per litter • ↓ litter weights and pup weights • ↓ % pups surviving until weaning	5	50 (No live pups delivered)		
	No effect on: • circulating levels of estradiol or progesterone • litter size • litter weights or calculated average pup weights, • % of pups surviving until weaning	50 (High dose; no effect)	–		
Other/Mechanistic					
CD-1 mice *Genistein:* 0 or 50 mg/kg bw/day genistein SC injection on PND 1–5	• Hypermethylation in *Nsbp1* promoter CG island (CG1) in an age dependent manner	–	50 (Single dose)	Tang et al., 2008 *(596)*	Limited (Parenteral)

Table 154 (continued)

Animal Model and Study Design	*Endpoints*	*NOEL*	*LOEL*	*Reference*	*Utility*
A v y mice 250 mg/kg genistein in diet before mating through pregnancy and lactation Assessment of heterozygous viable yellow agouti (*A* v y */a*) offspring	Shift in coat color phenotype towards the pseudo-agouti phenotype Reduced body weight in pseudo-agouti phenotype Hypermethylation	–	250 (Single dose)	Dolinoy et al., 2006 *(678)*	Limited (Oral)
	No effect on: • litter size • mean pup weight • percent survival • sex ratio	250 (High dose; no effect)	–		
A v y female mice Assigned to one of four modified diets for two weeks prior to mating with *A* v y */a* males and throughout pregnancy and lactation: 1) modified AIN-93 diet (corn oil substituted for soybean oil) 2) modified AIN-93 diet supplemented with 50 mg BPA/kg diet 3) modified AIN-93 diet supplemented with 50 mg BPA and 250 mg genistein/kg diet 4) modified AIN-93 diet supplemented with 50 mg BPA/kg diet and methyl donor compounds (4.3 mg folic acid, 0.53 mg vitamin B12, 5 g betaine, and 7.97 g choline chloride/kg diet)	Maternal dietary BPA significantly shifted the coat color distribution of genetically identical d22 *A* v y */a* offspring toward the yellow coat color phenotype. Maternal supplementation with methyl donors or genistein restored the coat color distribution in the BPA-exposed offspring to that observed in the controls.	–	250 (Single dose)	Dolinoy et al., 2007 *(679)*	Limited (Oral)
	No effects on: • litter size • survival • wean weight • genotypic ratio • sex ratio	250 (High dose; no effect)	–		
C57Bl/6 mice *Genistein:* 0, 25, 250, or 1250 ppm **[mg/kg feed] [Estimated by the study authors to provide genistein 0, 2, 20, or 100 mg/kg bw/day to a 25-g mouse]** In diet from GD14 to lactation via dam	Altered immune cell endpoints, e.g., various types of thymocytes and splenocytes (many did not display a consistent pattern of response) ↑ Male pup body weights ↑ Spleen weight in males	–	2[a]	Guo et al., 2006 *(682)*	Limited (Oral)
	↑ Maternal body weight ↑ Maternal spleen weight	2	250		

Table 154 (continued)

Animal Model and Study Design	*Endpoints*	*NOEL*	*LOEL*	*Reference*	*Utility*
ERE-tK-LUC transgenic male mice *Genistein:* 0 or 50 mg/kg genistein to dams Oral gavage on PND 4	↑Luciferase activity in: • liver • lung • heart • thymus • testis • brain of pups	–	50 (Single dose)	Montani et al., 2008 *(600)*	Limited (Oral)
ERE-tK-LUC mice *Genistein:* 0, 0.5, 5, and 50 mg/kg on PND 4 Oral gavage to lactating dam	↑Luciferase activity in: • the testes of male pups	5	50	Montani et al., 2009 *(605)*	Limited (Oral)

↑, ↓ = Significant increase, decrease.
[a]Did not display a dose-response.

Table 155. Experimental Studies with Developmental Toxicity Endpoints in Rats Exposed to Genistein, Daidzein, or Equol

Animal Model and Study Design	*Endpoints*	*NOEL*	*LOEL*	*Reference*	*Utility*
Growth, Reproductive System and Endocrine-Related Endpoints					
Sprague Dawley rat *Genistein:* 0, 5, 100, or 500 ppm in diet Multigeneration design: • during pregnancy and lactation • one half of male offspring continued on the genistein diets at weaning (G/G) • second half of male offspring were given control diets at weaning (G/C) **[Intakes (mg/kg bw/day) assumed to be similar to those in NCTR *(742)* of which this study was a part:** **• males: 0, 0.3, 7, 35** **• females: 0, 0.4, 9, 44** **• females during lactation: 0.7, 15, 78]**	F_1 males at PND 140: ↓ERβ protein in dorsolateral prostate (GC group only)	–	5 ppm	Dalu et al., 2002 *(628)*	Limited (Oral)
	F_1 males at PND 140: ↑serum DHT levels in F_1 males (both G/G and G/C groups)	5 ppm	100 ppm		
	F_1 males at PND 140: ↑serum testosterone levels (both G/G and G/C groups)	100 ppm	500 ppm		
Alderley Park rat *Genistein:* • 0, 0.2, or 2 mg/kg bw/day by SC injection during PND 1–6 • 4 and 40 mg/kg bw/day by gavage on PND 7–21 (SC doses were determined to be equivalent to gavage doses of 4 and 20 mg/kg bw/day)	• advanced vaginal opening • persistent vaginal cornification • uterotrophic response • ↓serum progesterone in females • ↓[b]ody weight in females	4	40 (Oral; PND 7–21) 20 (PND 1–6; equivalent to a SC injection dose of 2 mg/kg bw/day)	Lewis et al., 2003 *(243)*	High (Oral)
One part of the study examining SDN-POA dosed animals during the same period with SC and oral doses equivalent to 4 and 40 mg/kg bw/day by oral exposure.	• ↑SDN POA volume in females	4	40		

Table 155 (continued)

Animal Model and Study Design	*Endpoints*	*NOEL*	*LOEL*	*Reference*	*Utility*
CD®SD IGS rat *Genistein:* 0, 20, 200, or 1000 ppm **[mean: 1.7, 18, and 90 mg/kg bw/day]** GD 15 to PND 10 in diet	• ↓body weight in males at 11 weeks of age	–	1.7	Masutomi et al. *(640)*	Limited (Oral)
	• ↑ relative brain weight • ↑ relative pituitary weight	–	1.7[a]		
	• ↑ relative adrenal weight	1.7	18		
	• ↓absolute pituitary weight • ↓body weight gain in males on PND 21–42	18	90		
Sprague Dawley rat *Genistein:* 0, 5, 25, 100, 250, 625, and 1250 ppm through diet during pregnancy and lactation until PND 50 in offspring. **[Mean doses (mg/kg bw/day):** **• Pregnant dams: 0.31, 1.7, 5.7, 15, 34, 83** **• Lactating dams: 0.56, 2.8, 11, 30, 73, 138** **• Pups after weaning: ~0.6, 3.0, 12, 30, 72, 180]**	• accelerated vaginal opening • ↑ relative vaginal weight	–	Significant linear trend	Delclos et al., 2001 *(239)*	Limited (Oral)
	• hypertrophy of mammary alveoli and ducts in males at PND 50[b] • abnormalities of spermatogenesis	5 ppm	25 ppm		
	• histopathology in vagina at PND 50	250 ppm	625 ppm		
	• alveolar proliferation in mammary of females at PND 50 • ↑ rrenal tubule mineralization in males and females	100 ppm	250 ppm		
	• ↓dams delivering litters and ↓litter body weights at birth • ↓relative ventral prostate weight at PND 50 • prostate inflammation • histopathology in ovaries at PND 50 • ↓postnatal body weights in males and females	625 ppm	1250 ppm		
	• delayed eye opening and ear unfolding	625 ppm	1250 ppm (Significant linear trend)		
	No effect on: • maternal body weight during the lactation period • gestation length • litter size • proportion of live pups • sex ratio of pups • anogenital distance on PND 2 preputial separation • testicular sperm head or epididymal sperm counts	(High dose; no effect)	–		
Wistar (RORO) rats *Genistein:* 0, 5, 50, 100, 500 mg/kg bw/day GD 5–21 in diet	• ↑ post-implantation loss • ↓number of fetuses per female • ↓fetal body weight (per litter basis) • ↑ incidence skeletal ossification	100	500	McClain et al., 2007 *(612)*	Limited (Oral)

Table 155 (continued)

Animal Model and Study Design	*Endpoints*	*NOEL*	*LOEL*	*Reference*	*Utility*
Sprague Dawley rat *Genistein:* 0, 5, 100, 500 ppm genistein in diet Multigenerational design (mg/kg bw/day): • males: 0, 0.3, 7, 35 • females, average entire feeding period: 0, 0.5, 10, 51 • females, nonlactating: 0, 0.4, 9, 44 • females, lactating: 0, 0.7, 15, 78	↓ pup weight at birth (F_5) (no direct genistein treatment)	–	5 ppm	NCTR, 2008 *(637)*	Limited (Oral)
	• mammary gland hyperplasia in males (F_1, F_2, F_3) • ↓ male pup weight during lactation period (F_3)	5 ppm	100 ppm		
	• ↓ litter size (F_2) • disrupted estrous cycles: -↑ length or number of abnormal cycles (F_1) - increased cycle length (F_1, F_2) • ↓ body weight at vaginal opening (F_1, F_2 ,F_3) • accelerated vaginal opening(F_1, F_2) • delayed testicular decent (F_3) • ↓ anogenital distance in females (F_1, F_2) • ↓ anogenital distance males (F_1) • ↓ female pup weight during lactation period (F_1, F_3, F_4) • ↓ male pup weight during lactation period (F_2, F_4) • ↓ in feed consumption in adult females (F_0,F_1,F_4)	100 ppm	500 ppm		
	• ↓ litter size (F_1, F_3)	–	Significant negative linear trend		
	• ↓ anogenital distance in females (F_3)	–	100[a]		
	• ↓ male pup weight during lactation period (F_1)	?	5 ppm		
	No effect on: • mating, fertility, or pregnancy indices in any generation • duration of gestation • resorptions sites in animals that did not become pregnant • ovarian follicle count • sperm parameters	500 ppm (High dose; no effect)	–		
Sprague Dawley rat *Genistein:* 0, or 10 mg/kg bw/day GD14–birth[b] by gavage	• ↓ gonocyte proliferation in PND 3 testes • ↑ numbers of spermatogonia and preleptotene/leptotene spermatocytes on PND 21 (but not PND 60) • ↑ Leydig cell numbers on PND 21 and PND 60	–	10 (Single dose)	Thuillier et al., 2009 *(609)*	Limited (Oral)

Table 155 (continued)

Animal Model and Study Design	Endpoints	NOEL	LOEL	Reference	Utility
Wistar (RORO) rats *Genistein:* 0, 20, 150, or 1000 mg/kg bw/day GD 6–20 by gavage	• ↑ pup mortality	–	20 [a]	McClain et al., 2007 *(612)*	Limited (Oral)
	• visceral malformations (artery origin variant)	150	1000 (On litter incidence basis)		
	No effect on: • number of delivered pups • implantation or resorption sites • external malformation	1000 (High dose; no effect)	–		
Sprague Dawley rat *Genistein:* 0 or diet containing 0.5 g/kg genistein **[500 mg/kg diet]** **[Authors estimated intakes of 10 mg/day during pregnancy and 18.5 mg/day during lactation; this equals ~ 26–47 mg/kg bw/day based on the reported body weight of ~380 g in dams]**	No effect: • on pregnancy outcome • pup body weight during pregnancy, lactation, or after weaning • organ weights in offspring (uterus, ovary, testes, epididymides at PND 15, 49 and 75)	~26–47 (High dose; no effect)	–	Tousen et al., 2006 *(643)*	Limited (Oral)
Sprague Dawley dams *Genistein:* 0 or 5 ppm in diet GD 17 throughout the lactation period up to PND 70 in offspring **[Exposure in offspring estimated at ~0.68 mg/kg bw/day over the lifetime.]**	• changes in ovarian histology at PND 21 and 70 • ↓ body weight in PND70 females (continuous exposure to PND 70) • ↑ body weight in PND70 females (exposure prior to PND 21)	–	~0.68 [b] (Single dose)	Awoniyi et al., 1998 *(626)*	Limited (Oral)
	No effect in females on PND 70 on: • serum LH, FSH, 17β-estradiol, progesterone • ovarian or uterine weight	~0.68 [b] (Single dose; no effect)	–		
Sprague Dawley rat *Genistein:* 0, 25, 250, or 1250 ppm **[~ 2, 20, 200 mg/kg bw/day]** Diet from GD7 to weaning via dam and directly to offspring after weaning through PND 50	• hepatic CYP3A enzyme expression in males • altered formation of testosterone metabolites in liver microsomes, i.e., DHT/3-diol and 7α-OH	~2	≥~20 [a]	Laurenzana et al., 2002 *(639)*	Limited (Oral)
	• liver cytosolic ERα expression: - ↑ in females - ↓ in males • ↓ formation of testosterone metabolites in liver microsomes, i.e., 2α-OH and 16α-OH	~20	~200		

Table 155 (continued)

Animal Model and Study Design	*Endpoints*	*NOEL*	*LOEL*	*Reference*	*Utility*
Sprague Dawley rat *Genistein:* 0, 12.5, 25, 50, or 100 mg/kg bw/day PND 1 – 5.by gavage	• ↓ fertility index (genistein-treated females mated with untreated males) • polyovular follicles in weanling females • ↓ epididymal weight • ↓ body weights of males at 18 weeks of age (↓ body weights of females at 9 weeks of age)	–	12.5	Nagao et al., 2001 *(625)*	High (Oral)
	• ↓ normal estrous cycle (not dose dependent)	–	12.5[a]		
	• ↓ number of implants per litter	50	100		
	• ↓ body weights of both sexes at 1, 2, 3, 5, 7, 9 and 18 (males only) weeks of age • ↓ body weights of both sexes at 50 mg/kg bw/day from 5, 7, 9, and 18 (males only) weeks of age	–	100[a]		
	• hypertrophy of uterine myometrium	–	50[c]		
	• ↑ atretic follicles • ↓ corpus luteum 18 weeks • hypertrophy of luminal and glandular epithelial cells • ↓ in number of uterine glands	–	100[c]		
	No effect in males: • time to preputial separation, copulation or fertility • number of implants or number of resorptions in sired pregnancies • serum testosterone • epididymal sperm concentration • testicular histologic changes No effect in females: • in age at vaginal opening	100 (High dose; no effect)	–		
Long Evans rat *Genistein:* 0 or 15 mg/kg bw genistein GD 14 to PND 21 by gavage	• ↑ uterine progesterone receptor expression in glandular epithelial cells	–	15 (Single dose)	Hughes et al., 2004 *(627)*	Limited (Oral)
	No effect on: • uterine luminal epithelial cell height • uterine proliferation • ERα expression in uterine luminal or glandular epithelial cells • progesterone receptor expression in luminal epithelial cells	15 (High dose; no effect)	–		

Table 155 (continued)

Animal Model and Study Design	Endpoints	NOEL	LOEL	Reference	Utility
Pregnant Sprague Dawley rats *Genistein:* 0, 20, or 100 ppm genistein in diet **[0, 20, or 87 mg/kg bw/day]** Conception through postnatal life (GD1 to PND56)	• ↑ anogenital distance in females • ↓ weight at vaginal opening • ↑ uterus weight on PND 21 • ↑ relative testis weight on PND 21 • ↑ relative testis weight on PND 56 • ↓ ventral prostate weight	20	87	Casanova et al., 1999 *(634)*	Limited (Oral)
	No effect: on • implantation sites per dam • number of live pups per litter • litter weight at birth • proportion of males with retained nipples • age or weight at preputial separation • weight (absolute or relative) of the testis (PND 21 or 56) or ventral prostate (PND 56) as litter means	87 (High dose; no effect)	–		
Wistar rats Phytoestrogen-free (Ssniff SM R/M-H) and genistein rich diet (700 µg/g) during gestation and lactation: • F_1 female offspring weaned to diet of the dam • F_1 offspring assessed as intact juveniles and ovariectomized adults (OVX) **[The authors estimate exposures for the genistein rich diet were 73 mg/kg bw/day for juvenile and adult rats, respectively.]**	Juvenile: • ↑ uterine weight • ↓ IGF-1 and IGF-1R in uterus • ↓ ERα mRNA expression in uterus • ↓ PR mRNA expression in uterus • ↑ C3 expression in uterus	–	73	Moller et al., 2009 *(613)*	Limited (Oral)
	Adult (OVX): • ↓ C3 expression in uterus	–	93		
CD®SD IGS rat *Genistein:* 0 or 1250 ppm **[mean 147 mg/kg bw/day]** GD 15 to PND 11 in diet	• ↓ litter size • ↑ disrupted estrous cycles • ↑ endometrial • vaginal and mammary hyperplasia • atretic ovarian follicles	–	147 (Single dose)	Takagi et al., 2004 *(642)*	Limited (Oral)
	No effect on: • dam feed consumption or body weight • pup body weight on PND 3 • pup body weight gain during lactation period • pup survival to weaning • serum testosterone or 17β-estradiol on PND 3	147 (High dose; no effect)	–		

Table 155 (continued)

Animal Model and Study Design	*Endpoints*	*NOEL*	*LOEL*	*Reference*	*Utility*
Sprague Dawley rat *Genistein:* 0 or 5 (n=16) ppm **[0.12 mg/kg bw/day]** in feed from GD 17 to PND 21. At weaning, one half of genistein offspring were continued on genistein diet and remainder were provided control diet.	On PND 21: • ↓ serum LH and testosterone • ↓ pituitary mRNA expression of LH β-subunit On PND 130 in all males with prenatal/lactational exposure: • ↓ epididymis weight, • ↓ body weight On PND 130 in males with prenatal/lactational exposure only (not continuous exposure): • ↓ serum LH • ↓ testis weight No effect on: • serum T levels or pituitary mRNA expression of LH β-subunit on PND130 in all males with prenatal/lactational exposure • testicular spermatid count, serum FSH or pituitary mRNA expression of FSH β-subunit at any age	–	0.12[b]	Roberts et al., 2000 *(629)*	Limited (Oral)
Sprague Dawley rat *Genistein:* 0, 0.1, 10, or 100 mg/kg bw/day SC injection on GD 11–20	• Altered expression of 23 genes based on fetal testis or epididymis samples (pooled) in high dose group in a dose-related manner	10	100	Naciff et al., 2005 *(610)*	Limited (Parenteral)
	No effect: • maternal body weight • number live fetuses/litter • gross histopathological effects on fetal testis or epididymis	100 (High dose; no effect)	–		
Sprague Dawley rat *Genistein:* 0, 0.1, 1, or 10 mg/kg bw/day GD14–birth[b] by gavage	• ↑ Raf1 and Erk2 mRNA expression in PND3 testes	–	0.1[a]	Thuillier et al., 2009 *(609)*	Limited (Oral)
	No effect on PND3 on: • Erk1 and Mek1 mRNA expression • morphological changes in testes No effect in adult on: • fertility • levels or location of the phopho-ERK signals in testes • circulating testosterone levels	10 (High dose; no effect)	–		

Table 155 (continued)

Animal Model and Study Design	*Endpoints*	*NOEL*	*LOEL*	*Reference*	*Utility*
Lobund-Wistar rat *Genistein:* 0 or 250 mg/kg AIN-76 diet Four groups of genistein-exposed males: • control (Group A) • birth–PND35 (Group B) • PND90–11 months (Group C) • birth throughout life (Group D) N-methylnitrosourea (NMU) used to induce prostate cancer at PND70	Response to NMU in chemically-castrated rats implanted with a silastic capsule containing testosterone: • ↓ poorly differentiated prostatic tumors in Groups C and D (regulation of AR/Akt/PTEN axis suggested as molecular mechanisms)	–	250 mg genistein/kg AIN-76 diet (Single dose)	Wang et al., 2009 *(620)*	Limited (Oral)
Sprague Dawley rat *Genistein:* 0, 300, or 800 ppm **[mean exposures (mg/kg bw/day): 25 and 53 in dams, 30 and 84 in pups]** Diet during pregnancy and lactation and up to PND 90 in offspring	• ↓ birth weight of female offspring • accelerated vaginal opening	–	25	You et al., 2002 *(633)*	Limited (Oral)
	• ↓ body weight at PND21 and PND110 in males and females • ↑ pituitary weight in males • ↓ age at preputial separation when using body weight as covariate • ↑ mammary gland branching and PCNA immuno-staining of mammary epithelial cells in males	30	84		
	• no effect on liver, brain, ventral prostate, testis, or uterine weights at PND21 or PND110 • no histologic effects in male or female tissues	84 (High dose; no effect)	–		
Long Evans rats *Genistein:* 0, 10 mg/kg bw PND 0–3 by SC injection	• advanced vaginal opening • altered estrous cycling by 10 weeks • ↓ GnRH activation • ↓ AVPV KISS density	–	10 (Single dose)	Bateman and Patisaul, 2009 *(317)*	Limited (Parenteral)
	No effect: on: • ARC KISS fiber density	10 (Single dose)	–		
Sprague Dawley rat *Genistein:* 0, 0.1, 10, or 100 mg/kg bw/day GD 11–20 by SC injection	• altered expression of 227 genes based in pooled fetal ovary and uterine samples • 66 genes responded in similar pattern to ethinyl estradiol and bisphenol A	10	100	Naciff et al., 2002 *(607)*	Limited (Parenteral)
	No effect: on • maternal body weight, • number of live fetuses/litter, gross • histopathologic effects on ovary or uterus	100 (High dose; no effect)	–		

Table 155 (continued)

Animal Model and Study Design	*Endpoints*	*NOEL*	*LOEL*	*Reference*	*Utility*
Sprague Dawley rats *Genistein:* 0, 250 µg SC injection every 12h beginning on PND 1 for a total of 4 injections **[Assuming a Sprague Dawley rat pup weighs 7.5 g, each dose would be 33.3 mg/kg bw/d. Two injections per day for a total of 66.7 mg/kg bw/d genistein.]**	No effect on: • 5-HT fiber density • colabeling of VMNv1 ERα and HuC/D	66.7 (Single dose)	–	Patisaul et al., 2008 *(672)*	Limited (Parenteral)
Alpk:APfSD rat *Genistein:* 0, 0.2, or 2 mg/kg bw/day SC injection during PND 1–6 and 4 and 40 mg/kg bw/day by gavage on PND 7–21 (SC doses were determined to be equivalent to gavage doses of 4 and 20 mg/kg bw/day) One part of the study examining SDN-POA dosed animals during the same period with SC and oral doses equivalent to 4 and 40 mg/kg bw/day by oral exposure.	• advanced vaginal opening • ↓ body weight in females from PND 57 to 13 weeks • persistent vaginal cornification • ↓ serum progesterone	4	40	Lewis et al., 2003 *(243)*	Limited (Parenteral)
	• ↑ uterine weight at PND22 (following SC injection on PDN 1–6 only)	0.2	2		
	• ↑ SDN POA volume in females • ↑ uterine weight at PND22	4	40		
Wistar rat *Genistein:* 0 or 1 mg/day **[~19 mg/kg]** SC injection from PND 1–5	• early onset of vaginal opening • altered estrous cycling • ↓ body weight in females • ↓ ovarian weight • ↓ number of corpora lutea	–	~19 (Single dose)	Kouki et al. 2003 *(614)*	Limited (Parenteral)
		~19 (High dose; no effect)	–		
Wistar rat *Daidzein:* 0 or 1 mg/day**[~19 mg/kg]** SC injection from PND 1–5	• early onset of vaginal opening • altered estrous cycling • ↓ body weight in females • ↓ ovarian weight • ↓ number of corpora lutea	–	~19 (Single dose)	Kouki et al. 2003 *(614)*	Limited (Parenteral)

Table 155 (continued)

Animal Model and Study Design	*Endpoints*	*NOEL*	*LOEL*	*Reference*	*Utility*
CR CD rat *Genistein:* 0, 5, or 25 mg/animal/day **15 and 75 mg/kg bw/day]** SC injection from GD 16–20	• ↓ birth weight of females	15	75	Levy et al., 1995 *(669)*	Limited (Parenteral)
	• ↓ anogenital distance in males • delayed vaginal opening		15[a]		
	No effect: • volume of SDN-POA in either sex	75 (High dose; no effect)			
Sprague Dawley rat *Genistein:* 0 or 500 mg/kg bw SC injection on PND 16, 18, and 20	• ↑ uterine weight • ↑ serum 17β-estradiol • ↓ serum progesterone on PND 21 • ↓ ERα protein • ↓ androgen receptor protein • ↑ progesterone receptor protein (both isoforms)	–	500 (Single dose)	Cotroneo et al., 2001 *(215)*	Limited (Parenteral)
Brain and Behavior					
CD®(SD) IGS rat *Genistein:* 0, 20, 200, or 1000 ppm **[mean estimated intakes of 1.3–2.1, 13.7–23.0, and 66.6–113.1 mg/kg bw/day]** Diet from GD 15 to PND 10	No effect on: • proportion of pituitary cells staining for LH, FSH, and prolactin	66.6–113.1 (High dose; no effect)	–	Masutomi et al., 2004 *(641)*	Limited (Oral)
Sprague Dawley rat *Genistein:* 0, 5, 100, and 500 ppm **[0, 0.31, 5.7, 34 mg/kg bw/day]** Diet through gestation and lactation and in offspring until PND140	• ↑ calbindin-positive cells in SDN-POA in males	–	0.31	Scallet et al., 2004 *(674)*	Limited (Oral)
Sprague Dawley rat *Genistein:* 0 or 1000 ppm **[~66.6–113.1 mg/kg bw/day]** Diet from GD 15 to PND 11	No effect on gene expression of: • ERα, ERβ or progesterone receptor • steroid receptor coactivator in the hypothalamic pre-optic area	~66.6–113.1 (Single dose; no effect)		Takagi et al., 2005 *(675)*	Limited (Oral)

Table 155 (continued)

Animal Model and Study Design	*Endpoints*	*NOEL*	*LOEL*	*Reference*	*Utility*
CR CD rat *Genistein on PND 1-10:* 0, 100 or 1000 µg (0, 0.1 or 1 mg/day) **[mean≈12 and 117 mg/kg bw/day]** SC injection on PND 1–10	• non-dose-related ↑ in LH secretion	–	12 [a]	Faber and Hughes, 1991 *(665)*	Limited (Parenteral)
	• ↑ SDN-POA volume in females	12	117		
CR CD rat *Genistein:* 0, 0.001, 0.01, 0.1, 0.200, 0.4, 0.5, or 1.0 mg (0,1, 10, 100, 200, 400, 500, or 1000 µg) **[mean≈0.12, 1.2, 12, 23, 47, 58, and 117 mg/kg bw/day.]** SC injection on PND 1–10	• ↑ GnRH-induced LH secretion	–	0.12	Faber and Hughes, 1993 *(666)*	Limited (Parenteral)
	• ↑ SDN-POA volume	47	58		
Sprague Dawley rat *Genistein:* 0 or 250 µg/pup **[~100 mg/kg bw/day]** SC injection twice a day on PND 1 and 2	• ↑ in number of AVPV cells positive for tyrosine hydroxylase in males • ↓ number of AVPV cells positive for both tyrosine hydroxylase and ERα in females • ↓% of AVPV cells with ERα in both males and females	–	~100 (Single dose)	Patisaul et al., 2006 *(670)*	Limited (Parenteral)
Sprague Dawley male rat *Genistein:* 0 or 250 µg/pup **[~100 mg/kg bw/day]** SC injection twice a day on PND 1 and 2	• ↑ calbindin immunoreative cells in CALB-SDN in males • ↑ AVPV volume in males	–	~100 (Single dose)	Patisaul et al., 2007 *(671)*	Limited (Parenteral)
	No effect: • SDN or CALB-SDN volume • number of GnRH neurons, • Fos expression	~100 (High dose; no effect)	–		
Long Evans male rat *Genistein:* 0 or 10 mg/kg SC injection from birth (PND 0) to PND 3	No effect: • KISS immunoreactive neurons in the AVPV and ARC in males	10 (High dose; no effect)	–	Patisaul et al., 2009 *(673)*	Limited (Parenteral)
Long Evans male rat *Equol:* 0 or 10 mg/kg SC injection from birth (PND 0) to PND 3					

Table 155 (continued)

Animal Model and Study Design	*Endpoints*	*NOEL*	*LOEL*	*Reference*	*Utility*
Sprague Dawley rat *Genistein:* 0, 15, 150 or 300 mg (aglycone equivalent)/kg **[estimated maternal intake at 1–1.5, 10–15, and 20–30 mg/kg bw/day]** Diet during pregnancy and lactation via a cross-fostered dam. At PND 47, a portion of females were administered dimethylbenzanthracene (DMBA) to induce mammary gland tumors	• ↓ proportion surviving to 17 weeks in F_1 offspring following DMBA treatment on PND 47	1–1.5	10–15	Hilakivi-Clarke et al., 2002 *(653)*	Limited (Oral)
	• ↓ serum 17β-estradiol at 8 weeks • ↓ numbers of lobules • ↑ TEBs • ↑ mammary tumors in F_1 offspring following DMBA treatment on PND 47	10–15	20–30		
	No effect: • litter size • postnatal pup weight gain • tumor latency or multiplicity in F_1 offspring following DMBA treatment on PND 47	20–30 (High dose; no effect)	–		
Mammary Gland Development and Carcinogenesis					
Sprague Dawley rat *Genistein:* 0, 300, or 800 ppm Diet during gestation and lactation	• ↑ IGF-1 receptor	300 ppm	800 ppm	You et al., 2002 *(644)*	Limited (Oral)
	• ↑ branches, terminal end buds, and lateral buds in male offspring	300 ppm (P=0.06)	800 ppm		
	No effect: • serum prolactin	800 ppm (High dose; no effect)	–		
Sprague Dawley rat *Genistein:* 0, 25, or 250 ppm **[~0, 2.2, and 22 mg/kg bw/day]** Diet during pregnancy and lactation At PND50, all females were administered dimethylbenzanthracene (DMBA) to induce mammary gland tumors.	• ↓ DMBA-induced tumors • ↓ type 1 lobules	–	2.2	Fritz et al., 1998 *(209)*	Limited (Oral)
	• ↓ terminal end buds	2.2	22		
	No adverse effects on: • reproductive development in males or females • number of type II lobules • DNA labeling of mammary end buds or terminal ducts	22 (High dose; no effect)			
Sprague Dawley rat *Genistein:* 0, 300, 800 ppm genistein in diet from GD0 through gestation and lactation, offspring on same diet as dams after weaning (PND 22) until PND 90.	• moderate ↑ in glandular size and tissue density of mammary glands in males	–	300 ppm	Wang et al., 2006 *(662)*	Limited (Oral)
	• ↓ serum IGF-I (female)	300 ppm	800 ppm		
	No effect: • estradiol, testosterone, GH, FSH, LH or prolactin in females	800 ppm (High dose; no effect)	–		

Table 155 (continued)

Animal Model and Study Design	*Endpoints*	*NOEL*	*LOEL*	*Reference*	*Utility*
Sprague Dawley rat, *Dietary Genistein and Daidzein:* ≤ 5 ppm each of genistein and daidzein or 5, 100, or 500 ppm genistein Multigenerational design: • The F_0 generation was exposed from six weeks of age until termination • The F_1 and F_2 generations were exposed from conception through termination; • The F_3 generation was exposed from conception through weaning on PND 21 and then placed on the control diet until termination • The F_4 generation was not directly exposed to the prepared diets. A subset of male weanling F_1 and F_3 animals from the multigeneration studies were assigned to 2-year studies, three exposure durations were studied: • continuous exposure from conception through 2 years (F_1C-F_1 generation), • exposure from conception through PND 140 followed by control diet until termination ($F_1T140-F_1$ generation), • exposure from conception through weaning at PND 21 followed by control diet until termination (F_3T21-F_3 generation). All surviving animals were killed after two years and complete necropsies were performed.	• mammary gland hyperplasia in males in study terminated on PND 140 (F_1, F_2)	5 ppm	100 ppm	Latendresse et al., 2009 *(478)*	Limited (Oral)
	• mammary gland hyperplasia in males in 2-year study (F_1T140)	100 ppm	500 ppm		
	No effect: • mammary gland fibroma, • fibroadenoma, • neoplasia, or • pattern of feminization in male rats • ductal hyperplasia at any dose	500 ppm (High dose; no effect)	–		

Table 155 (continued)

Animal Model and Study Design	*Endpoints*	*NOEL*	*LOEL*	*Reference*	*Utility*
Sprague Dawley rat *Genistein:* 0 or 10 mg/kg bw/day genistein by SC injection on PND 2–8	Mammary glands showed evidence of: • lactation with cystic ductal dilatation, • atypical epithelial hyperplasia • microcalcifications *In situ* ductal carcinoma was identified in 2/5 animals examined	–	10 (Single dose)	Foster et al., 2004 *(649)*	Limited (Parenteral)
Sprague Dawley rats *Genistein:* 0 or ~0.1 mg/kg bw/day GD 15–20 by injection ("Experiment 1") Evaluated female offspring On PND 45, females were administered dimethylbenzanthracene (DMBA) to induce mammary gland cancer.	• ↑ incidence of mammary tumors in F_1 offspring following DMBA treatment on PND 45 • ↑ mammary ER protein content on PND 35 • ↓ protein kinase C activity on PND 45 (but not PND 21)	–	~0.1 (Single dose)	Hilakivi-Clarke et al., 1999 *(651)*	Limited (Parenteral)
	No effect: • number of pups/litter • PND 2 body weight • latency to tumor development • number of tumors per animal • number of tumors showing regular growth	~0.1 (High dose; no effect)	–		
Sprague Dawley rats *Genistein:* 0, ~0.5, or ~1.5 mg/kg bw/day by GD 15–20 by injection ("Experiment 2") Evaluated female offspring On PND 50, females were administered dimethylbenzanthracene (DMBA) to induce mammary gland cancer.	• ↓ body weight at PND 35 (but not earlier or later time points)	–	~0.5	Hilakivi-Clarke et al., 1999 *(651)*	Limited (Parenteral)
	• ↑ incidence of mammary tumors in F_1 offspring following DMBA treatment on PND 50 • ↑ mammary ER protein content on PND 50	~0.5	~1.5		
	No effect: • latency to tumor development • number of tumors per animal • number of tumors showing regular growth	~1.5 (High dose; no effect)	–		
Sprague Dawley rats *Genistein:* 0 or 20 µg genistein (dose level range of ~2 mg/kg bw on PND 7 to ~0.7 mg/kg bw on PND 20) Female offspring evaluated On PND 45, females were administered dimethylbenzanthracene (DMBA) to induce mammary gland cancer.	• ↓ tumor multiplicity • ↓ percentage of proliferating tumors in F_1 offspring following DMBA treatment on PND 45 • ↑ lobular differentiation, ↓ terminal end duct density, ↑ alveolar bud density	–	~0.7–~2 (Single dose)	Hilakivi-Clarke et al., 1999 *(652)*	Limited (Parenteral)
	No effect: • body weight gain • ER protein levels in mammary gland • incidence of mammary tumors • tumor latency in F_1 offspring following DMBA treatment on PND 45	~0.7–~2 (High dose; no effect)	–		

Table 155 (continued)

Animal Model and Study Design	*Endpoints*	*NOEL*	*LOEL*	*Reference*	*Utility*
Other/Mechanistic					
Sprague Dawley rat *Genistein:* 0, 5, 100, or 500 µg/g **[~0.75, 15, and 75 mg/kg bw/day for weanlings and ~0.5, 10, and 50 mg/kg bw/day in adult females]** In feed during gestation and lactation	• ↓ thyroid peroxidase activity in both sexes	–	~0.5–0.75	Chang and Doerge, 2000 *(242)*	Limited (Oral)
	No effect on: • serum levels of T3, T4, and TSH	~50–75 (High dose; no effect)	–		
Sprague Dawley rat *Dietary Genistein:* 0, 5, 100, or 500 ppm **[~0.0, 0.4, 8 and 40 mg/kg/day]** Multigenerational study design (F_0- F_4 with F_4 never exposed)	F_1 and F_2: • ↓ body weight of both sexes	0.4	8	Ferguson et al., 2009 *(636)*	Limited (Oral)
	• alterations in sexual dimorphism of intake of 3% sodium solution	40 (High dose; no effect)	–		
Sprague Dawley rat *Genistein:* 0, 25, 250, or 1250 ppm (0, 2, 20, and 100 mg/kg bw/day) In diet from GD 7, during gestation and lactation, until PND 77 in offspring.	• ↑ saline ingestion in both males and females	20	100	Flynn et al., 2000 *(667)*	Limited (Oral)
	• ↓ dam body weight • ↓ feed intake • ↓ offspring weight PND 42-PND 77	250	1250		
	No effect on: • gestational duration • total pups/litter • live pups/litter • sex ratio	1250 (High dose; no effect)			
Long Evans male rat *Genistein:* 0, 5, or 300 mg genistein /kg feed **[0.42 and 25 mg/kg bw/day]** Maternal exposure during pregnancy and lactation	• ↑ spleen count of total T cells • ↓ thymus counts of CD4−CD8− cells • ↑ thymus counts of CD4+CD8+ cells	–	0.42	Klein et al., 2002 *(684)*	Limited (Oral)
	• ↓ testosterone levels[a]	–	0.42[a]		
	• ↑ spleen counts of CD8+ cells	0.42	25		
	No effect on: • spleen counts of CD4+ cells, • thymus counts of CD4+CD8− and CD4−CD8+ cells • interleukin-4 or interferon-γ by cultured lymphocytes	25 (High dose, no effect)	–		

Table 155 (continued)

Animal Model and Study Design	*Endpoints*	*NOEL*	*LOEL*	*Reference*	*Utility*
Sprague Dawley rat *Dietary Genistein:* 0, 300, or 800 ppm **[~26 and ~69 mg/kg bw/day]** During gestation and lactation	• altered spleen natural killer cell activity in male and female offspring • ↓ number and percentage of CD4+CD8− thymocytes in male offspring • ↓ percentage of CD4+CD8− thymocytes in female offspring a	–	26	Guo et al., 2002 *(680)*	Limited (Oral)
	• ↓ body weight in male and female offspring • ↓ number and percentage of CD4−CD− thymocytes and percentage of CD4+CD8+ thymocytes in female offspring	26	69		
	No effect on: • spleen or thymus size in male or female offspring	69 (High dose; no effect)	–		
Sprague Dawley rat *Dietary Genistein:* 0, 25, 250, or 1250 ppm **[~2, 20, and 100 mg/kg bw/day]** GD 7 through lactation	• ↓ bone marrow DNA synthesis • ↑ colony forming units incubated with granulocyte macrophage colony • stimulating factor in bone marrow cells in females	–	2 [a]	Guo et al., 2005 *(681)*	Limited (Oral)
	• ↓ colony forming units incubated with granulocyte macrophage colony stimulating factor in bone marrow cells in males	2	20		
	• ↓ colony forming units incubated with macrophage colony stimulating factor in bone marrow cells in males	2	20 [a]		
	• ↓ recovered bone marrow cells in females	20	100		
	No effect on: • body weight of male rates	100 (High dose; no effect)	–		
Sprague Dawley rat *Genistein:* 0, 0.1, 1, or 10 mg/kg bw/day GD 14−PND 0 by gavage	• ↑ expression of PDGFα- and PDGFβ-receptor mRNA in testes of PND 3	–	0.1	Thuillier et al., 2003 *(608)*	Limited (Oral)
	• ↑ expression of PDGFα- receptor mRNA and PDGFβ-receptor mRNA in interstitium, central and peripheral seminiferous cords, and gonocytes at PND 3	–	10		

↑,↓ = Significant increase, decrease
[a] Did not display a dose-response
[b] The Expert Panel has limited confidence in the accuracy of the dose determination in this study.
c Reported in animals at lower dose levels, but not statistically analyzed.

Table 156. Experimental Studies with Developmental Toxicity Endpoints in Other Species Exposed to Genistein, Daidzein, or Equol

Animal Model and Study Design	*Endpoints*	*NOEL*	*LOEL*	*Reference*	*Utility*
Piglets *Genistein:* 0, 1, or 14 mg/L **[~0, 0.1–0.4, or 2–3 mg/kg bw/day]** In formula for 10 days	• ↓trefoil faction mRNA in stomach	–	~0.1–0.4	Chen et al., 2005 *(688)*	Limited (Oral)
	• ↓enterocyte proliferation • a trend for ↓enterocyte migration	~0.1–0.4	~2–3		
	No effect: • body weight • intestinal size • jejuna villous morphometric parameters • electrophysiological/clinical measurements (ion, glucose, glutamine transport in jejunum or ileum) • jejuna disaccharide, lactase, sucrose apoptosis in intestinal villi • ERα or ERβ expression in jejunum or ileum • phospho-src Tyr 416 protein expression in jejunum • trefoil faction mRNA in jejunum or ileum	~2–3 (high dose; no effect)	–		

↑,↓=Significant increase, decrease.

Table 157. Studies in Experimental Animals Exposed to Soy Formula, Soy Diets, or Other Soy Exposures During Development

Animal Model and Study Design	Endpoints	Reference	Utility
Growth, Reproductive System and Endocrine-Related Endpoints (Rat)			
Wistar rat Soy protein-based diet or same diet with isoflavones Removed from pre-mating through pregnancy and lactation	Soy protein-based diet: • ↑ activity during dark period at 3 months, but not 6 months • ↑ sensitivity to acetylcholine at 6 months (No effect at 3 months) • mesenteric arteries from females significantly more distensible at 6 months (unaffected at 3 months) No effect: • body weight in offspring • estrous cycling • total or HDL cholesterol or triglycerides in 3- or 6-month old offspring • blood pressure or heart rate • mesenteric artery diameter	Douglas et al., 2006 *(728)*	Limited (Oral)
Sprague Dawley rat fed soy and alfalfa-free diet Dams treated daily with: 0, 10, 50, 100, 150, 200 mg/kg bw/day soy isoflavones (80%, genistin: daidzin: glycitin = 13:5:2) PND 5 – 10 by gavage	Diets containing soy isoflavones < 150 mg/kg bw/day: • ↑ thickness of uterine lumen (≥ 100 mg/kg bw/day) • ↑ ovarian weight (≥ 100 mg/kg bw/day) • ↑ serum estradiol (≥ 50 mg/kg bw/day) • ↓ serum progesterone (100 and 200 mg/kg bw/day Diets containing soy isoflavones ≥ 150 mg/kg bw/day: • ↑ uterine weight • ↑ endometrial thickness • ↑ intensity of PCNA staining in ovary and uterus • ↓ PR mRNA expression in ovary • ↑ ER mRNA expression in ovary • ↓ ER mRNA expression in uterus No effect: • pup weight • number of uterine glands	Liu et al., 2008 *(692)*	High (Oral)
Long Evans rat Dams (n = 5/group) given phytoestrogen-containing diet (600 µg/g diet) or phytoestrogen-free fish meal-based diet throughout pregnancy and lactation. Offspring weaned to dam's diet, except at PND80 when one-half of phytoestrogen-containing diet fed offspring were switched to phytoestrogen-free diet.	F_1 offspring of dams fed phytoestrogen-containing diet: • delayed vaginal opening • ↓ body weight on PND 50 and 120 in male and female offspring • ↑ in prostate weight (absolute and relative) • ↑ SDN-POA in phytoestrogen diet males compared to control males and males switched from phytoestrogen diet to phytoestrogen-free diet at PND80	Lund et al., 2001 *(247)*	Limited (Oral)

Table 157 (continued)

Animal Model and Study Design	*Endpoints*	*Reference*	*Utility*
Long Evans rat Offspring of dams fed a diet of 0, 5, 50, 500, or 1000 ppm isoflavones GD 12 until weaning at PND 21	F_1 offspring of dams fed diets containing isoflavones: • At PND 5: ◦ ↑ body weight of male pups (5 and 50 ppm) ◦ anogenital distance (AGD) in male pups (5,50, and 500 ppm) **[AGD differences were not apparent when normalized for the pup body weights]** • At PND 21: ◦ ↑ body weight of males (5, 50 and 1000 ppm) ◦ ↑ testicular weight, absolute paired (≥5 ppm) ◦ ↑ testicular weight, relative (≥50 ppm) ◦ ↑ serum testosterone (5 ppm) ◦ ↓ serum testosterone (500 and 1000 ppm) • At PND 90: ◦ ↑ testicular weight, absolute (≥50 ppm) ◦ ↑ serum testosterone levels (1000 ppm) ◦ ↑ testicular testosterone secretion (1000 ppm) ◦ ↓ testosterone production per Leydig cell (500 and 1000 ppm) ◦ ↓ ERα mRNA levels of the ventral prostate No effect: • litter size • pup weight • sex ratios • PND 90 body weight • weights of the epididymis, dorsolateral and ventral prostate, bulbourethral glands and seminal vesicle PND 90	Akingbemi et al., 2007 *(694)*	Limited (Oral)
Wistar rat Dams given 15.5% soy-meal diet or soy-free diet prior to and during mating, pregnancy, and lactation (n=7–29, depending on endpoint)	F_1 offspring of dams fed soy-meal diet: • ↑ Sertoli cell nuclear volume on PND 18 • ↑ spermatocyte/Sertoli cell nuclear volume on PND 18 and PND 25 • ↓ body weight on PND 90–95 • ↓ testis weight on PND 90–95 • ↑ plasma FSH on PND 90–95	Atanassova et al., 2000 *(621)*	Limited (Oral)
Wistar rats Male offspring of dams fed a soy free or soy meal-containing diet during gestation and lactation. (Total isoflavone content in breeding and maintenance diets were 311 and 264 µg/g diet)	F_1 offspring of dams fed soy-meal diet: • ↑ PND 60 plasma LH and prolactin No effect: • reproductive organ weights PND 22 or PND 60	Gorski et al., 2006 *(695)*	Limited (Oral)

Table 157 (continued)

Animal Model and Study Design	*Endpoints*	*Reference*	*Utility*
Wister rat Fed casein-based diet or same diet enriched with 348 mg/g total isoflavones during gestation and lactation. (159 mg genistein, 156 mg daidzein, 33 mg glycitin) **[isoflavone intake of ~40 mg/bw/d]** At weaning, half of the F_1 pups from each maternal diet group were assigned to either the control diet or isoflavone enriched diet for a total of 4 treatment groups (C-C, C-IF, IF-C, IF-IF)	F_1 generation: • ↑ body weight of all rats receiving isoflavone enriched diet • ↑ uterine weight from 6 – 18 months, but not at 24 months (IF-C, IF-IF groups) • prolonged period of increasing BMD (IF-C, IF-IF groups) • ↑ total right femur BMD and distal femur metaphyseal zone BMD at 24 months (IF-C, IF-IF groups) • ↑ bone strength with age (IF-C, IF-IF groups) No effect: • age at which peak bone mass was achieved • femoral size • plasma osteocalcin • urine deoxypyridinoline • distal femur diaphyseal zone BMD	Mardon et al., 2008 *(729)*	Limited (Oral)
Sprague Dawley rats, two weeks of age **[feed not described]** Single daily dose of genistein (40mg/kg bw) SC injection for 3 days	• ↑ expression levels of genes in immature rat uterus (↑ 9.97%)	Hong et al., 2006 *(691)*	Limited (Parenteral)
Sprague Dawley rat Males (n = 6 – 12/group) from multigeneration study that continued male offspring on 1 of 6 diets given to their parents: • AIN-93G (soy-free, casein-based diet) • Control diet + soy protein (isoflavones 31.7 mg/kg feed) • Control diet + soy protein + soy-isoflavone concentrate (Novasoy®) at 36.1, 74.5, 235.8, or 1046.6 mg/kg feed	• ↑ Testis weight, PND 28 in 3 highest isoflavone groups (total isoflavone intake ≥ 2 mg/kg bw/day) • ↑ Serum testosterone and dihydrotestosterone, PND 120 in 2 highest isoflavone groups (total isoflavone intake ≥ 6.3 mg/kg bw/day) • ↑ Testicular 3β-hydroxysteroid dehydrogenase activity, PND 28, in 2 highest isoflavone groups **[Based on PND 28 testis weight NOAEL 1.2 mg/kg bw/day and LOAEL 2.0 mg/kg bw/day]** No effect at any time during the study on: • mean body weight • feed consumption	McVey et al., 2004a, 2004b *(697,698)*	Limited (Oral)
Sprague Dawley rat Pregnant rats (n = 15/group) given CRF-1, a soy-based diet (genistin at 10.2 mg/100 g diet and daidzin at 8.7 mg/100 g diet), or NIH-07, a soy-free diet.	F_1 offspring in soy-based diet (CRF-1) group: • ↑ PND 2 male pup body weight on PND 2 • ↑ PND 21 female pup body weight • ↓ anogenital distance adjusted for body weight in females.	Masutomi et al., 2004 *(699)*	Limited (Oral)

Table 157 (continued)

Animal Model and Study Design	*Endpoints*	*Reference*	*Utility*
Wistar-derived rat Dams (n=12/group) given assigned diet during pregnancy and lactation, and offspring weaned to assigned diet through PND 68. Diet assignments (dam/offspring) were: • RM3/RM1 (standard soy-based diets) • AIN-76/AIN-76 (soy- and alfalfa-free diet) • RM3/AIN-76A • Global 2016/Global 2016 (soy- and alfalfa-free diet) • Purina 5001/Purina 5001 (based on soybeans and alfalfa)	Effects compared to RM3/RM1 Control diet: *AIN-76A/AIN-76A* (soy free diet during pregnancy and lactation): ◦ ↑ age at testis descent ◦ ↓ age at preputial separation ◦ ↓ age at onset of vaginal opening • PND 26 females: ◦ ↑ body weight, uterine weight and vaginal weight • PND 68 males: ◦ ↑ body weight ◦ ↑ liver and kidney weight ◦ ↓ testis and epididymal weight • PND 128 males: ◦ ↑ body weight ◦ ↓ testis weight. • PND 140–144 females: ◦ ↑ body weight ◦ ↑ kidney weight ◦ ↓ uterine weight. *RM3/AIN-76A* (soy diet during pregnancy and soy free diet during lactation): ◦ ↓ age at testis No effect: • eye opening	Odum et al., 2001 *(622)*	Limited
Wistar rat Soy-containing diet, soy-free diet, and soy-free diet supplemented with methionine and L-lysine	• ↓ mating efficiency for F_0 animals • ↓ mean liver weight at 60 days in F_1 females • ↑ LH and prolactin at 60 days • difference in testosterone level not confirmed statistically No effect: • feed intake and body weight for F_0 generation • activity of hepatic enzymes • total cholesterol	Pastuszewska et al., 2008 *(700)*	Limited

Table 157 (continued)

Animal Model and Study Design	*Endpoints*	*Reference*	*Utility*
Growth, Reproductive System and Endocrine-Related Endpoints (Mouse)			
CD-1 mice F_0 dams during gestation and lactation and offspring after weaning were fed a soy-based diet (PMI 5008 or PMI 5001) or soy-free diet (PMI 5K96)	F_1 offspring of dams fed soy-based diet (PMI 5008 or PMI 5001) compared to soy-free diet (PMI 5K96): • ↓ serum estradiol levels in fetuses • ↑ birth weight of pups • ↓ PND 26 and PND 90 body weight (but no effect at PND 20) • ↓ uterine weight and epithelial cell height in PND 20 females in response to estradiol stimulation • ↓ uterine weight and epithelial cell height in PND26 females • later onset of fertility in females (46.5 versus 44.7 days) • ↓ prostate weight in males • better glucose clearance in adult males • ↑ testes, epididymides, seminal vesicles, liver and right kidney weight in males • ↓ weight of gonadal and renal fat pads in animals • ↓ serum leptin in PND 26 females and PND 90 in males and females No effect: • serum estradiol levels pregnant dams • PND 20 serum leptin • basal blood glucose levels and glucose clearance in adult females • mean pup weight or the number of pups produced by females on either diet	Ruhlen et al., 2008 *(706)*	Limited (Oral)
C3H mice F_0 generation fed a standard control diet **[assumed to contain some soy]** or a control diet + 2% (Soy Life®) supplement (ISF diet) during pregnancy and lactation F_1 fed the same diets as their parents post-weaning	F_1 offspring in ISF diet group: • accelerated vaginal opening • ↓ body weight, size and anogenital distance in females at the day of vaginal opening • ↓ size and body weight on PND 42 • loss of sexual dimorphism in DNA methylation in liver No effect: • pup sex ratio or litter size • anogenital distance in males or females on PND 42 • pup size or weight at PND 7, PND 14, or PND 21 • ERα promoter in liver • gender differences or within males or females in Acta1 in pancreas • lack of methylation of *c-fos* promotor	Guerrero-Bosagna et al., 2008 *(704)*	Limited (Oral)

Table 157 (continued)

Animal Model and Study Design	*Endpoints*	*Reference*	*Utility*
Han-NMRI mice Dams fed soy diet or soy-free diet during pregnancy and lactation Males in each litter were continued on the dam's diet or switched to the opposite diet Collection at 2 and 12 months of age (males)	2 month: • ↑ coagulating gland and combined prostate lobe relative weights in males fed a soy diet after weaning, following a soy-free diet given to the dam during pregnancy and lactation • ↑ relative weight of the prostate lobes when compared to males placed on a soy-free diet after weaning, regardless of which diet their dams had been fed • Soy feeding ↑ relative weight of coagulating gland (46%), dorsalateral prostate (81%) 12 month: • Soy feeding ↑ relative reproductive organ weights: ◦ ventral prostate (40%) ◦ coagulating gland (60%) ◦ dorsal prostate (63%) ◦ seminal vesicle (35%) ◦ testes (20%) No effect: • on body weight	Mäkelä et al., 1995 *(305)*	Limited (Oral)
Growth, Reproductive System and Endocrine-Related Endpoints (Non-Human Primate)			
Marmoset monkeys 7 sets of co-twins where one twin was fed a cow-milk based formula (SMA Gold™) and the other was fed soy infant formula (Wysoy®) from day 4 or 5 to day 35 to 45 Formula use was only partial as monkeys also nursed (estimated to result in isoflavone intake between 40 and 87% of the reported intake for a 4-month old human exclusively fed soy formula)	Effects in soy infant formula group up to 120–138 weeks of age: • ↑ testis weight • ↑ Sertoli cell and Leydig cell numbers/testis No effect: • body weight • weights of thymus, spleen, prostate, seminal vesicles, pituitary • penile length • timing of onset of puberty • plasma testosterone • fertility	Tan et al., 2006 *(709)*	Limited (Oral)

Table 157 (continued)

Animal Model and Study Design	*Endpoints*	*Reference*	*Utility*
Marmoset male monkeys Co-twins where one twin was fed a cow-milk based formula (SMA Gold™) and the other was fed soy infant formula (Wysoy®) from day 4–5 to day 35–45 (n=7–14 twin pairs, depending on age of evaluation) Formula use was only partial as monkeys also nursed (estimated to result in isoflavone intake of ~1.6–3.5 mg/kg bw/day)	Effects in soy infant formula group up to PND 35–45: • ↓ plasma testosterone at PND 35–45 • ↑ Leydig cells/testis at PND 35–45 No effect: • body weight • formula intake • plasma testosterone PND 18–20 • testis weight at PND 35–45 • Sertoli cell and germ cell number/testis at PND 35–45 • 17α-hydroxylase/C17–20 lyase staining in testis at PND 35–45 • immunopositive cells for β-subunit LH or FSH in pituitary sections at PND 35–45	Sharpe et al., 2002 *(708)*	Limited (Oral)
Mammary Gland Development and Carcinogenesis			
Sprague Dawley rat Fed AIN-93G diet in which soybean oil was replaced by corn oil and the protein source was either casein, whey, or soy-protein isolate containing isoflavones 430 mg/kg diet (genistein 276 mg/kg diet and daidzein 132 mg/kg diet) **[~20.4 genistein mg/kg bw/day].** At weaning, the F_1 and F_2 offspring were fed the same diets as their dams and continued to receive the diets throughout their lifetimes. At PND 50, rats gavaged with 80 mg/kg DMBA and the development of palpable mammary tumors assessed	F_1 and/or F_2 females in soy-protein group: • ↓ body weight gain at 8 weeks (both generations) • accelerated vaginal opening (PND 37 vs PND 38) • ↑ latency to develop mammary gland tumors, in terms of the number of post-treatment days for 50% of rats to develop at least 1 tumor (F_1 and F_2, compared to casein) • ↓ latency to develop mammary gland tumors, in terms of the number of post-treatment days for 50% of rats to develop at least 1 tumor (F_2, compared to whey) • ↓ percentage of rats with at least 1 mammary tumor at the end of the study (F_2) • ↑ tumors/rat compared to whey diet group (F_2) No effect: • relative organ weight • estr first tumor • % of rous cycle • successful breeding • sex ratio • litter size • time toats with adenocarcinoma or tumor with intraductal proliferation • median tumor volume • tumors/rat compared to casein diet group	Hakkak et al., 2000 *(713)*	Limited (Oral)

Table 157 (continued)

Animal Model and Study Design	*Endpoints*	*Reference*	*Utility*
Sprague Dawley rat Female F_1 offspring of F_0 parents fed AIN-93G diets containing either casein or soy-protein isolate from GD 4 to lactation. At weaning, females were weaned to the diet of the dam. At PND 50, rats IV injected with 50 mg/kg MNU and followed until 115 days following MNU treatment	Female F_1 offspring of F_0 parents fed soy-protein isolate: • ↓ incidence of rats with at least 1 mammary gland tumor • ↑ tumor latency in rats fed the soy-protein diet • ↓ incidence of ductal carcinoma *in situ* but a higher incidence of infiltrating ductal carcinoma • ↑ PR and HER-2/neu (involved in cell proliferation) gene expression • ↓ serum progesterone levels • ↑ apoptotic cells in an MCF-7 culture incubated with sera No effect: • cell proliferation or apoptosis • serum estradiol	Simmen et al., 2005 *(717)*	Limited (Oral)
FVB mice fed: • (I) control diet (F_1 females) • (II) 270 mg isoflavone/kg diet throughout lactation (PND 0–21) • (III) 270 mg isoflavone/kg diet from weaning through the end of the study • (IV) E2 during the lactation period	Effects in isoflavone-treated groups (Groups II and/or III): • ↑ branching in mammary trees (Groups II/III) • ↑ in overall branching in juvenile mammary gland (PND 28, Groups II/III) • ↑ TEB after postweaning isoflavone treatment (Group III) • ↓ TEBs at PND 42–43 after postweaning (Groups III) No effect: • PNDs 70–73 • levels of plasma estradiol • gene expression profiles • gene expression level when comparing postweaning isoflavone exposure to E2 exposure.	Thomsen et al., 2006 *(719)*	Limited (Oral)
Brain and Behavior			
Long Evan rat Pregnant dams fed a diet containing phytoestrogen glycosides 600 µg/g diet (phyto-600) or a phytoestrogen-free diet throughout the gestation and lactation period. Offspring were weaned to the same diet as the dam. On PND 80, half the offspring fed phyto-600 were switched to a phytoestrogen-free diet for 40 days prior to collection at PND 120.	Effects in phyto-600: • ↓ body weight, absolute prostate weight and relative prostate weight on PND 75 • ↑ AVPN nucleus volume in males switched to the phytoestrogen-free diet on PND 80 No effect: • brain aromatase activity in APVN • APVN in females unaffected by change in diet, PND 110 • sexual dimorphism in AVPV volume	Lephart et al. 2001 *(232)*	Limited (Oral)

Table 157 (continued)

Animal Model and Study Design	*Endpoints*	*Reference*	*Utility*
Long Evans rat Dams given phytoestrogen-containing diet (600 µg/g diet) or phytoestrogen-free fish meal-based diet throughout pregnancy and lactation. Offspring weaned to dam's diet.	F_1 offspring of dams fed phytoestrogen-containing diet: • ↓ PND 50 body weight • ↑ PND 50 water intake • ↑ time spend in open arms and number of entries into the open arms of elevated plus-maze apparatus	Lund and Lephart, 2001 *(723)*	Limited (Oral)
Sprague Dawley rats Dams fed a phytoestrogen-containing diet (200 µg/g diet, genistein 95 µg/g, glycitin 17 µg/g, and daidzein 82 µg/g) or phytoestrogen-free diet from GD 0–20	Fetuses of dams fed phytoestrogen-containing diet: • ↓ calbindin protein expression in the medial basal hypothalamus and preoptic area in females (and displayed more of a sexual dimorphism compared to animals in the phytoestrogen-free diet group) No effect: • litter size • anogenital distance • fetal body weight • calbindin protein expression in males	Taylor et al., 1999 *(724)*	Limited (Oral)
Other/Mechanistic			
Female New Zealand rabbits (8 months old) Fed: • (Group 1) soy and alfalfa free diet • (Group 2) soy and alfalfa free diet plus 5 mg/kg bw/dayay of soy isoflavones (ISGO5) • (Group 3) a soy and alfalfa free diet • (Group 4) diet containing 18% soy meal plus a cornstarch placebo (S+) (~ 13 mg/kg bw/dayay of isoflavones). Pups weaned at 5 weeks old and fed same diet as mother.	• ↓ weight in ISF_20 group at 13 weeks of age • ↓ food consumption in ISF_20 group at 13 weeks of age No effect: • organ weights • histopathological differences in the testes or accessory sex glands • age that males exhibited the mounting reflex or in the time of reaction	Cardoso and Bao, 2007 *(711)*	Limited (Oral)
Rabbits Pregnant does fed: • (1) control diet (a soy and alfalfa-free diet) • (2) control diet supplemented with 10 mg/kg bw/dayay of soy isoflavones • (3) control diet supplemented with 20 mg/kg bw/dayay isoflavones • (4) a diet containing 18% soy meal	No effect: • on dam feed intake, duration of gestation, or litter size • offspring body weight, histopathology of the testes or epididymides • organ weights • feminization or demasculinization • male sexual behavior • ejaculate volume • sperm motility, vigor, concentration	Cardoso and Bao, 2008 *(712)*	Limited (Oral)

Table 157 (continued)

Animal Model and Study Design	*Endpoints*	*Reference*	*Utility*
Outbred CD-1 mice fed AIN-93G diets devoid of estrogenic compounds F_1 offspring treated by SC injection with: • corn oil • daidzein (2mg/kg bw/dayay) • genistein (5 mg/kg bw/dayay) • daidzein plus genistein (2mg daidzein + 5 mg genistein/kg/day) • diethylstilbestrol (2 mg/kg bw/dayay) Administered each morning from PND 1 to PND 5 (5 doses).	Variety of effects on bone measures: • bone mineral content • bone mineral density • micro architecture that were interpreted as "positive" effects although the combination of genistein and daidzein was not considered to have a greater benefit than the individual treatments No effect: • body weight	Kaludjerovic et al., 2009 *(683)*	Limited (Parenteral)
Sprague Dawley rats *Experiment 1:* • Pregnant rats fed AIN-93G (CAS) diet. • On PND 15 litters assigned to: ◦ control (CAS) diet, ◦ SPI +diet (286 mg/kg genistein and 226 mg/kg daidzein, 80 mg total isoflavones/kg bw/day) ◦ SPI- diet ◦ C+G diet (250 mg/kg genistein) ◦ C+G diet (250 mg/kg daidzein) • Pups fed to PND 33. *Experiment 2:* • Male rats received: ◦ AIG-936 (CAS diet), ◦ Western casein diet, or ◦ Western SPI+ diet from PND 24 to 64	Effects in SPI diet group compared to casein control: • ↑ Serum genistein levels of male rats in the C+G diet group (2700 nmol/L) than the SPI+ diet group (808 nmol/L) at PND 50 • ↓ body weight • ↓ serum IGF-1 concentrations • ↓ altered gene expression pattern with an overall pattern of increased expression of hepatic genes regulated by PPARα, PPARγ, and LXRα and decreased expression of genes regulated by SREBP-1c • ↑ weight gain, percent body fat, percent liver weight • ↑ serum insulin concentration • ↑ insulin sensitivity • ↑ expression of hepatic genes regulated by PPARα, PPARγ, and LXRα • ↓ expression of genes regulated by SREBP-1c.	Ronis et al., 2009 *(702)*	Limited (Oral)

Table 157 (continued)

Animal Model and Study Design	*Endpoints*	*Reference*	*Utility*
Piglets (n = 16 from 4 litters) randomly assigned 48% soybean meal-based liquid diet or cow-milk based diet. Protein source continued after weaning until 56 days of age.	• ↓ body weight gain at 28 days of age • intestinal villi shortened at 28 but not 56 days of age • ↑ IgG titers to soy in both age groups of piglets fed soybean compared to milk-protein diets No effects of diet on: • serum concentrations of zinc, selenium, iron, and copper at 28 and 56 days of age. • on blastogenic responses of peripheral or intestinal lymphocytes collected at either age and cultured with purified soy proteins, phytohemagglutinin, or pokeweed • No significant differences in skin thickness following intradermal injection of soy or milk proteins were observed between the soybean- and milk-diet groups at 27 and 55 days of age	Li et al., 1990 *(738)*	Limited (Oral)
Adult female cynomolgus monkeys (n = 19) and offspring (n = 25) Ged typical American diet (TAD) diet with protein derived from soy or TAD diet with the protein derived from casein-lactalbumin. Offspring continued TAD diet fed to mothers after weaning. TAD soy diet provided isoflavone aglycone-equivalent dose of 180 mg/monkey/day (~40 – 50% genistein, 40% daidzein, and 10 – 15% glycitein).	• ↑ serum isoflavone concentration in TAD soy diet monkeys (Offspring consuming TAD casein had similar isoflavone concentrations to adults consuming TAD casein, but offspring consuming TAD soy had higher concentrations than the adult females consuming TAD soy) • ↓ fructosamine concentrations • ↓ body weight at 1 and 2 years of age (offspring) • ↑ insulin in males compared with females • ↑ HDLC in females compared with males • ↓ glucose AUC and faster disappearance of glucose (offspring) • ↓ insulin responses to glucose challenge No effect: • body weight (adults) • TC, TG, apoB-C, glucose (adults) • body weight at birth (offspring) • HDLC concentration • fasting glucose and insulin concentration	Wagner et al., 2009 *(710)*	Limited (Oral)

the studies on growth have not detected differences in growth of full-term infants fed soy and cow-milk formula, but most of the studies had small sample sizes *(518; 523; 524; 530; 532)*. Agostoni *et al.*, 2007 *(541)* reported better short-term weight gain in infants with immunoglobulin E-mediated cow's milk allergy who were fed a rice or casein hydrolysate formula compared to soy formula. Some studies reported that infants given soy formula had transiently lower bone-mineral content than infants fed cow-milk formula *(520)* or human milk *(510)*; however, other studies reported that serum concentrations of bone accretion-related hormones and minerals in soy formula-fed infants demonstrated the appropriate compensatory response to the vitamin and mineral content of the food source *(516; 517; 524)*. Reports in very low birth-weight and preterm babies suggest that soy formula may not support adequate growth and bone development in these groups *(522)*. Soy infant formula is not marketed for use in preterm infants, and the AAP recommends against its use in this group.

Studies comparing soy- and cow milk-formula feeding in infants with familial predispositions to allergic disease did not detect an advantage of one formula over another *(517; 550; 554)*, but most of these studies have demonstrated that breast feeding was superior to the use of either formula. A series of studies by Chandra *et al.*, 1989a, 1989b, 1991, 1997 *(542-546)* related to allergic disease were considered by the Expert Panel but classified as no utility for the evaluation because the validity of the papers has been challenged and the scientific integrity cannot be confirmed.

Case reports suggest that hypothyroid infants on oral thyroxine therapy fed soy formula may exhibit less efficient absorption of the thyroxine from the intestine. This effect has been attributed to fecal wastage with decreased enterohepatic circulation *(570; 572; 573)*. Although the addition of iodine and reduction in fiber content of soy formulas is a partial solution, 1 paper noted that the problem may occur if formula intolerance increases stool frequency *(570)*. Conrad at al., 2004 *(571)* reported that infants with congenital hypothyroidism (CH) fed soy formula had a prolonged elevation of TSH levels compared to infants with CH fed non-soy formula suggesting that infants with CH fed soy formula may require higher levothyroxine doses to achieve normal thyroid function tests.

A case-control study of premature thelarche used 130 subjects from 552 potentially eligible girls *(576)*. Age-matched controls were recruited, consisting in some but not all instances of friends of the cases. Parents were interviewed with regard to family history and possible exposures, including the use of soy formula. Multivariate analysis did not show a significant relationship between premature thelarche and soy-formula feeding. When the analysis was restricted to girls with onset of premature thelarche before 2 years of age, a significant association with soy-formula feeding was identified (OR 2.7, 95% CI 1.1–6.8). Other significant factors included maternal ovarian cysts and consumption of chicken. Consumption of corn was protective.

Strom *et al.*, 2001 *(32)* identified 952 adults who had participated as infants in a controlled, non-randomized formula trial. There were 248 adults who had been given soy formula and 563 who had been given cow-milk formula. The subjects were asked about height, weight, sexual maturation, education level, and reproductive outcomes. No formula-related differences in height, weight, body-mass index, or sexual maturation were identified. Women who had been given soy formula had longer menstrual periods (mean 0.37 days longer, 95% CI 0.06–0.68 days) and there was a soy formula-associated increase in the risk of experiencing severe menstrual discomfort (RR 1.7, 95% CI 1.04–3.00 compared to no or mild discomfort, unadjusted). After adjustment for multiple comparisons, these findings were no longer statistically significant (more than 30 different endpoints were evaluated).

In a study comparing women with and without breast cancer by Boucher *et al.*, 2008 *(575)*, the point estimates of the odds ratios were less than unity. However the variability of these estimates indicate that feeding soy formula was not statistically significantly associated with developing breast cancer (soy formula only during first 4 months of life: OR=0.42, 95% CI=0.13–1.40; soy formula only during 5-12 months of age: OR=0.59, 95% CI=0.18–1.90).

3.6.2 Experimental Animal Studies on the Individual Isoflavones Found in Soy Formula

Studies reporting the most sensitive and apparently treatment-related developmental effects are summarized in **Table 154** for oral and parenteral exposures in mice, **Table 155** for oral and parenteral exposures in rats, and **Table 156** for animal studies in other mammalian species. In these tables, dose levels have been converted to mg/kg bw. In general, the most complete information was available from parenteral exposure studies in mice and oral exposure studies in rats. In cases where doses were converted to mg/kg bw/day values, ranges were often estimated over periods of gestation or lactation or in different stages of the offspring's life.

3.6.2.1 Pre- and Postnatal Survival, Growth, and General Development Endpoints

Oral exposure studies conducted in rats suggested that genistein exposures can adversely affect prenatal endpoints such as growth and possibly survival. The most consistent and sensitive prenatal endpoint was lower pup birth weight, which was reported at ≥300 ppm genistein (≥25 mg/kg bw/day in dams during pregnancy) administered in diet *(633)*; lower pup birth weight was seen in other studies at higher dose levels *(637; 667)*. A lower number of mated dams delivering litters was reported in 1 study at 1250 ppm genistein in diet (83 mg/kg bw/day in dams during pregnancy)*(239)*. Decreased live litter size was reported in 2 studies at ≥500 ppm in diet (44 mg/kg bw/day in dams during pregnancy) *(637; 642)*. In rats gavaged with genistein during the neonatal period, a lower pregnancy rate was observed at ≥12.5 mg/kg bw/day and lower numbers of implants were observed at 100 mg/kg bw/day *(625)*. None of the studies assessed structural malformations.

Oral exposure studies examining postnatal development in rats suggested that genistein exposures can result in lower and delayed growth. In well-designed multiple dose-level studies, lower pup weight or weight gain during the lactation period were observed with exposures in diet given to dams from early-to-mid gestation through lactation *(239; 633; 637)*. The lowest effect level in these studies was ≥100 ppm genistein (≥11 mg/kg bw/day in dams during lactation) in the NCTR multigeneration study *(637)*. Lower body weight was also shown with gavage dosing of pups with ≥100 mg/kg bw/day during the lactation period *(625)*. One multiple-dose level study with gestational and lactation exposure reported trends for developmental delay and significant delays in eye and ear opening at 1250 ppm genistein (≥138 mg/kg bw/day in dams during lactation) *(239)*. None of the studies reported adverse effects on postnatal survival.

3.6.2.2 Reproductive endpoints

3.6.2.2.1 Mouse

Female mice

There is some evidence that genistein affects endocrine-mediated endpoints in female mice. Disrupted estrous cycles were seen in mice exposed neonatally by oral *(217)* or sc *(585; 592)* dosing. Early

vaginal opening was observed following fetal *(585)* or PND 15-19 *(595)* exposure via sc injections to dams or pups , respectively. A lower number of live pups was observed in mice exposed neonatally by oral *(217)* or sc dosing *(592; 594)*. Following neonatal-prepubertal sc exposures, abnormal uterine and ovarian histology was observed *(334; 595)*. Additionally, multiple oocyte ovarian follicles were observed following neonatal sc exposure *(591; 593)* although the relevance to humans is unclear.

Male mice

A shorter anogenital distance, adjusted for body weight, was observed in 21 day old male offspring of dams exposed to 10 mg/kg bw/day genistein from GD 12–PND 20 by gavage *(604)* suggesting an anti-androgenic effect of treatment. Since anogenital distance was not consistently evaluated in the available mouse studies it is not clear if this is a reproducible effect of genistein in mice. Anogenital distance was not altered consistently in male rats exposed to genistein during development (see below). A higher incidence of hypospadias in male fetuses was observed following oral exposure of dams to 0.17 mg/kg bw/day genistein between GD 13-17 *(587)*. No other studies in mice or rats in which male offspring were exposed to genistein during late gestation have reported a higher incidence of hypospadias. Hyperplasia was reported in the prostate and seminal vesicles of adult mice that had been treated sc with 500 mg/kg bw/day genistein on PND 1-3 *(603)*. Conversely, the relative weights of prostate lobes were lower in adult mice from the same study following sc treatment with ≥50 mg/kg bw/day genistein on PND 1-3 *(603)*. No effects on sperm count or motility were reported and *in vitro* fertilization was not lower following oral or sc exposures of dams or developing offspring to genistein *(602)*. There were also no consistent effects on testis weights or testicular morphology following oral or sc exposure of male mice *(601; 602)*.

3.6.2.2.2 Rat

Female rat

Oral exposure studies suggest that genistein can affect endocrine-mediated reproductive endpoints in female rats. Trends or significant effects on accelerated vaginal opening were observed in a number of studies by oral *(239; 243; 628; 633; 634; 637)* or sc injection *(614)* exposure during the prenatal or neonatal period. The lowest genistein effect level for alterations in vaginal opening was ≥300 ppm (≥30 mg/kg bw/day in pups) in the study of You *et al. (633)*. One study reported a higher number of polyovular follicles in 21-day-old rats following direct gavage dosing with ≥12.5 mg/kg bw/day genistein during the neonatal period *(625)*. Abnormal estrous cycles were reported by oral *(637; 642)* or sc *(614)* exposure routes. Smaller litter sizes were seen with oral transplacental exposure *(637; 642)*. Direct ovarian and uterine effects (including morphological changes) were observed in a number of studies with oral or sc exposure during the prenatal or neonatal period *(215; 239; 243; 607; 613; 614; 625; 626; 634; 642)*. Effects of genistein on uterine weight are reported in Chapter 2 which describes *in vitro* and *in vivo* studies of estrogenic activity. No other consistent effects on female reproductive organ weights were reported.

Male rat

The majority of effects of genistein reported in the reproductive system of male rats were on the prostate and male mammary glands (see below for a discussion of the mammary data). Chronic inflammation of the dorsolateral prostate on PND 50 was reported following mid-gestational, lactational, and postweaning exposure to dietary genistein at 180 mg/kg bw/day (dose in offspring) *(239)*. Lower ventral prostate weight was observed at PND 50-56 following dietary exposure to ≥87 mg/kg bw/day genistein (dose in offspring) *(239; 634)*. A limited number of studies examined effects of genistein exposure during development on hormone levels, and the results were variable in males and females.

There were no consistent effects on testicular and epididymal weights or testicular morphology in male offspring. Lower gonocyte proliferation at PND 3, higher numbers of spermatogonia and preleptontene/leptontene spermatocytes were observed at PND 21, and higher Leydig cell numbers at PND 21 and PND 60 were observed in male offspring of dams given 10 mg/kg bw/day genistein from GD 14 to birth by gavage *(609)*. With the exception of one study reporting greater severity of abnormal spermatogenesis on PND 50 in male rats given a dietary dose of 180 mg/kg bw/day genistein from GD 7−PND 50, which may have been related to the peripubertal status of the rats *(239)*, no other studies reported adverse effects on sperm count and/or motility at genistein doses up to 35 mg/kg bw/day genistein in diet with exposure during gestation, lactation, and postweaning *(637)* or 100 mg/kg bw/day by gavage during the neonatal period *(625)*. A multigeneration study that included exposures in males during prenatal and postnatal development reported smaller litter sizes at dietary doses of 51 mg/kg bw/day in dams, but no adverse effects on fertility in males at dietary doses up to 35 mg/kg bw/day genistein *(637; 741)*.

The majority of oral exposure studies reported no effects on anogenital distance after adjustment for body weight in male and female rats following genistein doses up to 180 mg/kg bw/day (doses in offspring) administered during gestation, lactation, and/or postweaning development *(239; 612; 633; 640)*. Furthermore, a shorter anogenital distance, adjusted for body weight, was reported in F_1 male and F_1 and F_2 female rats on PND 2 in a multigeneration study that included exposures during prenatal and postnatal development at dietary doses of 51 mg/kg bw/day *(637; 741)*. In contrast, longer anogenital distance at birth was reported for female rats exposed to a diet containing both genistein and daidzein (16 mg genistein and 14 mg daidzein per 100 g of feed) during gestation, lactation, and postweaning development *(634)*.

Most studies reported no effect on age at preputial separation at dietary doses up to 180 mg/kg bw/day genistein in offspring *(239; 634; 640; 642)* or 100 mg/kg bw/day by gavage *(625)* following direct or indirect exposure during the gestational, lactational, and/or postweaning periods. However, a dietary dose of 84 mg/kg bw/day genistein (in offspring) enhanced the effect of methoxychlor in delaying preputial separation when administered GD 1 - PND 21 *(633)*.

Effects of developmental exposure to genistein on pup weight were variable; however, a number of studies reported an association with lower fetal body weight *(612)*, lower body weight of male and/or female pups during lactation *(628; 637)*, and lower postweaning or adult body weight or weight gain in males and/or females *(239; 625; 626; 637; 640)* at oral doses of 35-180 mg/kg bw/day genistein.

3.6.2.3 Mammary Gland Development and Carcinogenesis

Mouse

No effects on mammary growth or differentiation in adult mice were reported following gavage exposure of their dams with up to 10 mg/kg bw/day during mid gestation through lactation (Fielden *et al.*, 2002 *(645)*). Two studies examined the effects of genistein injection in CD-1 mice. In a single dose study, Hilakivi-Clarke *et al.*, 1998 *(646)* administered 0.7-0.8 mg/kg bw/day on GD 15-20 to pregnant dams and measured morphological changes in the mammary gland at various postnatal times in the female offspring. These investigators found significantly higher mammary gland epithelial area on PND 35 (but not PND 25 or PND 46; and a persistently higher density of terminal end buds (TEBs) when measured on PND 35 and 45. Padilla-Banks *et al.*, 2006 *(647)* administered 0, 0.5, 5,

or 50 mg/kg bw/day on PND 1-5 and reported higher mammary gland ductal elongation at 6 weeks. The physiological relevance of effects they observed at the highest exposures 50 mg/kg bw/day is uncertain. However, lower numbers of TEBs (at 6 weeks) reduced alveolar development (LOEL 5 mg/kg bw/day), and increased mammary gland PR and ERβ and lower ERα expression levels also were reported (occurred at LOEL 0.5 mg/kg bw/day). Neither of these studies included assessments of mammary carcinogenesis but higher numbers of TEBs is generally associated with an increased susceptibility to mammary neoplasia (Hilakivi-Clarke *et al.*, 1999 *(651)*). The changes reported in the normal mammary glands at the higher doses and later exposures by Padilla-Banks *et al.*, 2006 *(647)* are generally associated with a reduced susceptibility to mammary carcinogenesis. The use of single doses and the route of administration (injection) are limiting factors for several of the studies noted above.

Rat

Hypertrophy/hyperplasia of mammary structures was reported following dietary genistein exposure during periods including mid-to-late gestation and/or the neonatal stage, at doses ≥100 ppm in males (≥5.7 mg/kg bw/day in dams and 7–12 mg/kg bw/day in offspring) *(239; 478; 637)* and 1250 ppm in females (≥83 mg/kg bw/day in dams and 180 mg/kg bw/day in offspring) *(239; 642)*. Higher ductal branching of the mammary glands was also reported in 22 day-old male rats that had been exposed to 84 mg/kg bw/day genistein (dose in offspring) from GD 1–PND 22 *(633)*.

Smaller numbers of terminal end buds/ducts and greater numbers of lobules in mammary glands were reported in adult female rats that received genistein by sc injection during development *(323; 654; 655)*. Inconsistent effects on mammary structures were observed in adult rats that were exposed to genistein through diet during the developmental period, with one study reporting lower numbers of terminal end buds and lobules *(209)* and another study reporting no effects on mammary structures of females *(644)*. Numbers of chemically induced mammary tumors were lower in rats sc treated during postnatal development with 500 mg/kg bw/day genistein *(323; 654; 655)*. In the only oral dose study examining the effects of genistein exposure on chemically-induced mammary tumors, dietary exposure to ≥25 ppm genistein (~2.2 mg/kg bw/day) during gestation and lactation reduced dimethylbenzanthracene-induced tumorignicity in adult females *(209)*.

Males

A multigenerational, dose response study in male rats reported mammary gland hyperplasias at the termination of the study on PND 140 in both the F_1 and F_2 generations (LOEL=100 ppm; *(478)*. Up to the highest dose tested (500 ppm; all doses administered p.o.) no effects were reported with respect to mammary gland neoplasia or ductal hyperplasia. You *et al.*, 2002 reported greater branching and numbers of TEBs and lateral buds in the mammary glands of male offspring (dams exposed orally to 0, 25, or 80-0 ppm during pregnancy and lactation) that were of borderline significance (P=0.06) at 300 ppm and significant at an 800 ppm exposure *(644)*. Dietary exposure from GD 0 through gestation and lactation (offspring on same diet after weaning) to 0, 300, or 800 ppm genistein produced greater glandular size (LOEL=300 ppm) in male offspring *(662)*.

Females – GD 0 through lactation exposures

Fritz *et al.*, 1998 *(209)* reported no effects on chemically-induced mammary carcinogenesis in rats exposed to dietary genistein (0, 2.2, 22 mg/kg bw/day during pregnancy and lactation). Exposure

(parenteral) to 1.5 mg/kg bw/day on GD 15-20 (but not 0.5 mg/kg bw/day) was associated with a higher incidence of mammary tumors (DMBA; ~40 mg/kg bw; PND 50) and greater mammary gland of total ER binding (measured on PND 50) *(651)*. Hilakivi-Clarke, 2002 *(653)* administered 1-1.5, 10-15, or 20-30 mg/kg bw/day during pregnancy and lactation and reported lower numbers of lobules, higher numbers of TEBs, and higher numbers of mammary tumors (DMBA; ~50 mg/kg; PND 47) only with the highest dose. In a single dose study (0.1 mg/kg bw/day, parenteral, GD 15-20) Hilakivi-Clarke *et al.*, 1999 *(652)* reported a higher incidence of mammary tumors in F_1 offspring (DMBA; ~50 mg/kg; PND 45) associated with a higher number of mammary ER binding sites (measured on PND 35) and lower protein kinase C activity (PND 45 but not PND 21)−experiment 1; and a higher incidence of mammary tumors and shorter latency−experiment 2.

Females – postnatal exposures

Parenteral administration of 0 or 10 mg/kg bw/day genistein on PND 2-8 showed evidence of lactation with cystic ductal dilation, atypical epithelial hyperplasia, microcalcifications and *in situ* ductal carcinomas (2/5 animals examined) in a study reported by Foster *et al.*, 2004 *(649)*. Hilakivi-Clarke *et al.*, reported lower tumor multiplicity (DMBA; ~50 mg/kg; PND 45), greater lobular development, lower TEB density, increased alveolar bud density following parenteral administration of genistein (LOEL 0.7-2 mg/kg bw/day)*(652)*. The use of single doses, the route of administration (parenteral), and the occasional use of small numbers of animals are limiting factors for many of the studies noted above.

Studies of genistein/soy exposure conducted in rodents were largely designed to test hypotheses related to how the physiology of the normal mammary gland might be modified to affect susceptibility to mammary carcinogenesis. Few were designed to test a standard toxicological hypothesis and with the rigor required for detailed toxicological analysis, e.g., dose response features are often not well represented among the study designs. The route of administration and dosing varies significantly across studies, with some using exposures or regimens that are not readily seen to be relevant to human exposures. Hence, many studies were either excluded or judged to be of limited utility.

Despite the limitations, there are some potential trends evident in the studies. The potential for an effect of the timing of exposure on outcome is a relatively consistent observation when viewed across all studies. While the precise role of each developmental time of exposure is unclear, there is evidence suggesting that *in utero* exposure only may increase later susceptibility to mammary carcinogenesis, while later exposure (birth to onset of sexual maturity) may be protective. Exposure only during normal adulthood (after sexual maturation) may have no major effect on susceptibility to carcinogenesis in the mammary gland. The limited data on cross-generational effects are also interesting but insufficient to allow for any definitive conclusion.

It is difficult to derive any overall conclusion on a clear association of soy exposure with mammary carcinogenesis. When compared with the limited human data from the meta-analyses, the animal model data support the potential for a modestly protective effect for some exposures, e.g., childhood/adolescent exposure might have a small reduction in risk. A protective effect of exposure in pre-existing breast cancers is potentially more problematic. The limited but possibly more consistent data from *in vitro* and *in vivo* studies using low (perhaps more physiologically relevant) exposures in breast cancer models show that genistein can act as an estrogen. Consistent with this activity, a small number of studies suggest that genistein may reduce responsiveness to antiestrogens or aromatase inhibitors.

These data likely explain the increasing practice among medical oncologists to advise against soy/isoflavone consumption for breast cancer patients, as further reflected in the guidelines proposed by the American Cancer Society (see *http://www.cancer.org/docroot/ETO/content/ETO_5_3x_Soybean.asp*). For the moment, this would appear to be a reasonable precaution.

3.6.2.4 Brain and behavior

Rat

Treatment with genistein has been reported to increase the size of the sexually dimorphic nucleus of the preoptic area (SDN), as quantified by Nissl staining, in adult female rats. Lewis *et al.*, 2003 *(243)* observed a greater size of the SDN in adult females following sc administration of 2 mg genistein/kg bw during PND 1-6 (day of birth was PND 1) followed by oral consumption of 40 mg genistein/kg bw/day (LOEL and NOEL: 40 and 4 mg/kg bw/day oral, respectively) from PND 7-21. The dose of genistein increased the SDN in females relative to control, however it was not as effective as a high dose of DES (10 µg/kg bw/day) which produced a female SDN equal to the size of the control male *(243)*. Faber *et al.*, 1991, 1993 conducted two studies in which the SDN was examined *(665; 666)*. In the first study, pups were injected (sc) from PND 1-10 with 0, 0.1 or 1 mg of genistein, including positive controls DES and Zearalenone. Both DES and 1 mg genistein (LOEL 12 mg/kg bw/day oral) increased the size of the SDN in females to that of males *(665)*. In the second study *(666)*, a range of lower doses (0, 0.001, 0.01, 0.1, 0.2, 0.4, 0.5, or 1 mg sc) were administered daily from PND 1-10. In this study, only the two highest doses of genistein (0.5 and 1 mg; equal to body weight corrected doses of 58 and 117 mg/kg bw/day sc, respectively), increased the SDN in adult females. The only other study that simply used Nissl staining to examine the SDN did not find any effects of much higher doses of genistein In contrast, sc injections of genistein at 5 or 25 mg (corresponding to 15 and 75 mg/kg bw/day) did not affect the size of the SDN; however, the sc injections were given to gestating dams on GD 16-20 so the level of genistein the embryos were exposed to is unclear *(669)*.

Another way to visualize the SDN is by using immunocytochemistry to stain the region for Calbindin d28k. Scallet *et al.*, 2004 *(674)* fed dams a casein-based diet supplemented with 0, 5, 100 or 500 ppm genistein (corresponding to 0, 0.31, 5.7, and 34 mg/kg bw/day; LOEL >0.31 mg/kg bw/day oral) from conception through lactation, and the F_1 offspring were continued on these diets until examination at PND 140. Male offspring exposed to the genistein treatments had a larger SDN as compared with the control males, while there was no effect of treatment in female offspring. Patisaul *et al.*, 2007 *(671)* injected pups on PND 1-4 with 0.25 mg genistein (LOEL ~100 mg/kg bw/day) and found higher Calbindin-SDN volumes in genistein-treated males in adulthood. In contrast, to the Calbindin result, there was no effect of genistein on the Nissl stained SDN in the female rats relative to controls. These two studies *(671; 674)* used Sprague Dawley rats that were fed a base diet of soy-free chow. All the studies in the previous paragraph involved rats fed standard chow (which contains phytoestrogens and soy), except one study *(243)* which referenced diet R&M No 3 (Special Diet Services Ltd, Stepfield Witham, Essex) containing 100-110 ppm genistein. In addition, the other studies used different strains of rats; CD *(665; 666; 669)* and Alderley Park rats *(243)*. Thus, strain and diet might play major roles on the differentiation of the SDN and its sensitivity to genistein. In addition the data suggest that the classical "biomarker" of the SDN, calbindin d28k, is not always informative for the responses of all the neurons in the SDN. Finally the finding that genistein exposure can increase the SDN in males but

not in females is not as predicted if genistein was acting only as an estrogen agonist and speaks to a complexity in the actions of genistein.

Another sexually dimorphic nucleus that has been examined is the anteroventral periventricular nucleus of the hypothalamus (AVPV), which is typically larger in females than in males. Patisaul *et al.*, 2006 *(670)* evaluated the AVPV by measuring tyrosine hydroxylase (TH) levels by immunocytochemistry. Pups received a total of 4 injections sc of genistein (0.25 mg; LOEL ~100 mg/kg bw/day sc) with one injection administered every 12 hours for the first two days after birth. On PND 19, the brains were collected and TH positive cells were analyzed in the AVPV. Treatment lowered the number of TH cells co-expressing ERα in the female brain closer to male cell number values, while genistein increased the number of TH cells in the AVPV of males closer to female values. These data suggest that when endogenous estrogens are present (in the PND 1-2 male) genistein has an anti-estrogenic effect.

Patisaul *et al.*, 2009 *(673)* reported there was no effect of genistein on the number of kisspeptin (KISS) immunoreactive cells in the AVPV of adult males following injections of genistein (10 mg/kg; NOEL 10 mg/kg bw/day) from PND 1-4. In another study *(317)* female Long Evans rats were treated for the first four days after birth with estradiol, genistein (same dose as above), either an ERα or ERβ specific agonist, or equol. All treatments were in oil given by sc injections. In adulthood, females were ovariectomized and given sequential estradiol then progesterone to promote LH release. The numbers of co-labelled cfos/GnRH cells was lower in the early genistein treated females than controls. Also, kisspeptin immunoreactivity was lower in the AVPV by the early genistein treatment. The ERβ agonist had less of an effect on these measures than the ERα. This suggests that genistein can have its actions via ERα.

Effects of genistein on other aspects of the hypothalamic-pituitary-gonad axis have been examined by few studies. Masutomi *et al.*, 2004 *(641)* fed pregnant rats a diet containing genistein (0, 20, 200, 1000 ppm; NOEL range of 66.6-113.1 mg/kg bw/day oral) from GD 15 until PND 10. When pituitaries were analyzed for gonadotropin subtypes at 11 weeks of age, no effect of genistein was found. In another study, pregnant dams were fed a diet of 0 or 1000 ppm genistein (LOEL 113.1 mg/kg/bw/day oral) from GD 15 to PND 10. On PND 10, the hypothalami (including the SDN) were collected and RNA was used for real time RT-PCR determinations of PR, ERα, ERβ and SRC-1 *(675)*. There were sex differences in ERα (females>males) and PR (males>females), but genistein had no effect on any of the measures.

3.6.2.5 Other endpoints and mechanistic studies

One study reported lower thymocyte subsets and changes in natural killer cell activity in rats on PND 22 following dietary exposure of dams during gestation and lactation *(680)*. A second study found changes in thymocyte numbers suggesting augmented cell-mediated immunity in PND 70 rats the dams of which had been given dietary genistein during pregnancy and lactation *(684)*. The inconsistency in the data detracts from the utility of the developmental immunotoxicology data set.

Most of the mechanistic studies used high subcutaneous doses. The most widely studied mechanistic effect was expression of estrogen, progesterone, and androgen receptors in reproductive organs of rodents. In studies with gestational and lactational exposure of dams, effects on offspring were only observed with sc dosing. Lower ERα and androgen receptor and higher progesterone receptor expression

were observed following sc injection of rats with 500 mg/kg bw/dose on 3 days during the late lactation period *(215)*. In mouse ovary, higher ERα expression was noted at lower doses (≤10 µg/day) and lower expression was noted at a higher dose (100 µg/pup/day) following neonatal sc exposure *(591)*.

Two studies in which mice were sc injected with genistein in the neonatal period reported reductions in expression of testicular ERα (≥7 mg/kg bw) and androgen receptor (≥71 mg/kg bw/day) *(601)*, but no effect was reported following maternal dietary genistein exposure during gestation and lactation at up to 10 mg/kg bw/day *(604)*. In a study examining estrogen receptor expression in rats exposed through diet from gestation through weaning or adulthood, results were somewhat variable in different generations and often not dose-related, but lower expressions were noted for ERα (≥25 ppm) and ERβ (≥100 ppm) *(628)*. A transgenic mouse model has been engineered to express luciferase activity as a reporter of ER transcriptional activity, and studies have shown that oral gavage of genistein to dams on PND 4 results in lower luciferase activity in the male offspring confirming lactational exposure to genistein is able to activate ER *(600; 605)*.

Results of estrogen or progesterone receptor expression in mammary gland following oral or sc exposure in rats were variable, with no obvious patterns related to dose or period of exposure observed *(644; 648; 650)*. One series of studies was interpreted by authors as suggesting that acute sc exposure of immature animals to genistein 500 mg/kg bw results in increased differentiation of immature terminal end buds, leading to a greater number of lobules, thought to be more resistant to carcinogens, during adulthood *(658)*. It appeared that the effects were mediated through ERs, which regulate both progesterone receptor and EGF receptor. Upregulation of EGF receptor (EGFR) in immature rats does not occur through tyrosine phosphorylation. EGFR is down-regulated in adult rats, and it has been hypothesized that a less active EGF-signaling pathway in adulthood suppresses mammary cancer development. A third study reported upregulated expression of *BRCA1*, a tumor suppressor gene involved in DNA damage repair, following sc exposure of rats to genistein during the lactational period *(648)*.

3.6.3 Experimental Animal Studies of Soy Formula or Other Soy Exposures During Development

Almost 30 experimental animal studies are summarized in **Table 157**, one study was judged to have high utility and the rest were found to be of limited utility for the evaluation process.

3.6.3.1 Growth

The effect of exposure to dietary soy on pup body weight was inconsistent among studies. In a study by Masutomi *et al.*, 2004 *(699)* using Sprague Dawley rats, female offspring in the soy-diet group showed a higher body weight on PND 21, greater body weight gain between PND 10−21, and shorter weight-adjusted anogenital distance compared to the control group. Male offspring in the soy-diet group showed a higher body weight on PND 2 and greater body weight gain between PND 10 - 21. In this study exposure to dietary soy was likely limited to the dams since they were given a soy-based or a soy-free diet until PND 10, prior to the time when pups will have eaten maternal feed. Higher pup body weights prior to weaning were also reported in male rats by Akingbemi *et al.*, 2007 *(694)*, and in both female and male rats by Odum *et al.*, 2001 *(622)*. In the studies by Akingbemi *et al.*, 2007 and Odum *et al.*, 2001, rats were exposed to soy during gestation and lactation. The body weight was greater in male offspring on PND 5 and PND 21 *(694)*, and in both male and female offspring from birth to PND 13 *(622)*.

When body weight was measured after weaning, the opposite effect was observed with numerous studies reporting a lower adult body weight associated with developmental exposures to soy-based diets (e.g., lower body weight in male and female rats at PND 50 and PND 120 *(247)*, lower body weight in male rats at PND 75 *(232)*, lower body weight in male rats at PND 90 *(621)*, decreased body weight in male rats at PND 280 *(695)*). Odum *et al.*, 2001 *(622)*, observed higher body weight in male and female rats from birth to PND 13, no effect around weaning (i.e., PND 13-21), and decreased body weight in males from PND 26 to PND 128 and in females from PND 26 to PND 140 when a standard soy-based diet was given to the dams during gestation and lactation, and then to offspring postnatally. Ruhlen *et al.*, 2008 *(706)* reported the same pattern in CD-1 mice, with higher birth weight, no effect of body weight around weaning (PND 20 and 26), and lower body weight with lower renal and gonadal fat pad weights at PND 90 for F_1 offspring of CD-1 mice exposed to a soy-based diet when compared to a animals fed a soy-free diet.

There are also several studies that report no change in adult body weight following developmental exposure to a soy-based diet in rats *(694; 697; 700)*. Mardon *et al.*, 2008 *(729)* reported body weight in rats given a soy based diet with observations covering longer exposure periods than most studies, and including data on 2 year old rats. There was no effect of soy consumption on body weight in rats from birth to 18 months of age; however, there was a higher body weight and body fat mass observed in 24 month-old rats when a soy-based diet was given to the dam during gestation and lactation, and/or to the pups from weaning to 24 months of age *(729)*.

There was no effect of soy intake on body weight at PND 35-45 in marmoset monkeys given soy-based formula from days 4 or 5 until weaning at PND 35-45 compared to controls fed a cow's milk based formula *(708; 709)*. There was also no effect on body weight from birth to 6 months of age in cynomolgus monkeys when a soy-based diet was given to pregnant monkeys and the offspring were fed the same soy-based diet *(710)*. In contrast, body weight was lower in older cynomolgus monkeys (1 and 2 years of age) exposed to the soy-based diet developmentally and with continued maintenance *(710)*.

Female

In all experimental animal studies exposure to soy proteins was via the oral route. However, findings were inconsistent between the studies. In the mouse, one study reported an acceleration in the age at vaginal opening (Guerrero-Bosagna *et al.*, 2008 *(704)*). In another study, Ruhlen *et al.*, 2008 *(706)* reported a delay in puberty (measured as the age at which the female mice produced their first litter). Both studies involved prenatal and postnatal exposure to soy proteins in the feed. Body weight was lower in both studies, and there was less in fetal circulating 17β-estradiol *(706)*. In addition, estradiol-stimulated uterine weight was lower in PND 20 females on the high soy diet relative to those on the control diet.

In Wistar and Sprague-Dawley rat strains, exposure to soy in the feed during the prenatal and postnatal periods was linked to early vaginal opening, greater body weights and uterine weights *(622)* (675,639, 568) relative to rats on soy-free diets, with the exception of the females on the Global 2016 diet (soy-free) in which vaginal opening onset was not different than in the soy-fed controls *(622)*. Body weight was lower and vaginal opening onsets were delayed, relative to controls, in Long Evans rats exposed to isoflavones in the diet during the prenatal and postnatal period *(247)* and 264). Circulating 17β-estradiol serum levels were higher in neonatal rats exposed via the dam on PND 5-10 *(692)*.

The expert panel is of the opinion that, due to inconsistent findings, it is difficult to draw conclusions about effects of soy protein on female growth, reproductive system, and endocrine-related endpoints. However, a consistent effect of genistein and soy protein in female mice and rats was an acceleration of the timing of vaginal opening. This finding may have clinical relevance for timing of puberty in girls.

Male

Several rodent studies compared reproductive endpoints in male offspring of dams given soy-based and soy-free diets during pregnancy and lactation with pups weaned to their dam's diet *(247; 305; 622; 697; 698)*. The results reported in these studies were inconsistent. Odum *et al.*, 2001 *(622)* reported advancement of pubertal landmarks in males (and females). Testis weight was reported to be lower at PND 90-95 by dietary soy in one study *(621)* and higher by dietary soy in rats at PND 28 *(697)*. Higher testicular weight was reported in 68 and 128 day-old offspring of rats given a soy-based diet during pregnancy, lactation, and postnatally; epididymal weight was also higher at PND 68 *(622)*. Greater weight of the testes, epididymides, and seminal vesicles were also reported in adult offspring (i.e., at PND 90) of pregnant mice fed a soy-based diet during gestation, lactation, and given to offspring post-weaning *(706)*. Greater testicular and epididymal weights were reported at PND 160 in male offspring of rats fed a soy meal-containing diet; however testicular and epididymal weights were not altered in older (i.e., PND 280) or younger rats (PND 22 or 60) from the same study *(695)*. Several studies have reported lower adult prostate weight in mice *(706)* or rats *(232; 247)* exposed to soy- or phytoestrogen-based diets during gestation, lactation, and postnatally when compared to animals raised on a soy-free diet. Other studies have reported no change in prostate weight following exposure during gestation, lactation, and postnatally in rats *(694; 695)* or during lactation in marmosets *(709)*. These differences may reflect different isoflavone contents of the diets used in different studies or other differences in the composition of the feed. The study of McVey *et al.*, 2004 *(697; 698)* permits a dose-response evaluation of dietary isoflavone supplementation and developmental effects. These investigators used a soy-free diet to which was added soy protein and soy isoflavones at 5 exposure levels (plus the soy-free diet control). The most sensitive endpoint was higher testis weight on PND 28 in rats that were exposed during pregnancy, lactation, and weaning to a dietary-isoflavone intake ≥2 mg/kg bw/day; however, it is not known if this is related to changes in spermatogenesis. The higher testis weight did not show a dose-response relationship.

The study of Sharpe *et al.*, 2002 *(708)*, extended by Tan *et al.*, 2006 *(709)* involved the feeding of marmoset co-twins with either soy infant formula or a cow-milk formula. The authors expected the use of co-twins to permit paired analyses and to decrease variability between animals assigned to different treatments. The study authors reported a lower in plasma testosterone on PND 35–45 with an higher in testis weight and in numbers of Sertoli and Leydig cells at 120–138 weeks of age. Interpretation of this study was complicated by design since the offspring were also nursed by their mothers on a daily basis.

3.6.3.2 Mammary gland development and carcinogenesis

In FVB mice, Thomsen *et al.*, 2006 *(719)* fed either control diet (F_1 females) - Group 1, 270 mg isoflavone/kg diet throughout lactation (PND 0-21) - Group 2, 270 mg isoflavone/kg diet from weaning through the end of the study - Group 3, or E2 during the lactation period - Group 4. Effects in isoflavone-treated groups (Groups 2 and/or 3) included an greater branching in mammary trees; lower overall branching in juvenile mammary glands (PND 28; Groups 2/3); higher numbers of TEBs after

postweaning isoflavone treatment (Group 3); and lower TEBs at PND 42-43 after postweaning (Group 3). The higher number of TEBs would be expected to increase susceptibility to carcinogenesis but the later reduction would eliminate/reverse this effect. Thus, timing of carcinogen exposure would likely affect the apparent estimates of changes in the mammary gland's susceptibility to carcinogenesis.

Hakkak *et al.*, 2000 *(713)* fed Sprague Dawley rats AIN-93G diets in which soybean oil was replaced by corn oil and the protein source was either casein, whey, or soy-protein isolate containing isoflavones 430 mg/kg diet (genistein 276 mg/kg diet and daidzein 132 mg/kg diet) [~20.4 genistein mg/kg bw/day]. At weaning, the F_1 and F_2 offspring were fed the same diets as their dams and continued to receive the diets throughout their lifetimes. Accelerated vaginal opening was observed on days PND 37 vs PND 38. At PND 50, rats were gavaged with 80 mg/kg DMBA and the development of palpable mammary tumors assessed. The authors reported a shorter latency to develop mammary gland tumors (F_1 and F_2) and an greater number of tumors/rat compared to the whey diet group (F_2). Also in Sprague Dawley rats, Simmen *et al.*, 2005 *(717)* fed female F_1 offspring of F_0 parents fed AIN-93G diets containing either casein or soy-protein isolate from GD 4 to lactation, at weaning females were weaned to the diet of the dam. At PND 50, rats iv injected with 50 mg/kg MNU and followed until 115 days following MNU treatment. The investigators reported a lower mammary tumor incidence (rats with at least 1 mammary gland tumor), and a longer tumor latency, in rats fed the soy-protein diet. While the incidence of ductal carcinoma *in situ* was lower, a higher incidence of infiltrating ductal carcinoma was observed.

Three primary tumorigenesis endpoints are usually measured. Latency (time to appearance) and incidence (number of animals with one or more mammary tumors) are likely reasonable measures with respect to the human disease. Multiplicity (number of tumors/animal) may be less widely applicable but is potentially relevant as contralateral breast cancers arise in some women. While the data from the rodent studies are mixed in their general outcomes with respect to changes in mammary gland differentiation and cancer susceptibility, several of the exposures appear to induce changes that increase susceptibility to carcinogenesis. These may be estrogenic effects, as E2 can produce the same outcomes and other markers of estrogenicity are evident, e.g., accelerated vaginal opening, and increased TEBs. While the weight of evidence tends to favor adverse effects from exposure, it is difficult to arrive at a compelling determination of the lifetime effects on mammary cancer risk. Some of the changes that could affect risk are time dependent and modifications that could either increase or decrease risks are reported.

3.6.3.3 Brain and behavior

In three studies the main manipulation was phytoestrogens in the diet. The only behavioral study was conducted in Long Evans rats *(723)* with life-long (starting prior to conception) exposure to phytoestrogen-free or phytoestrogen-containing (600 µg/g) diet. As adults, males and females were tested in the elevated plus maze for anxiety-like behavior. Rats on the phytoestrogen-containing diet were less anxious and more active in the maze than the rats not exposed to phytoestrogens. Lephart *et al.* 2001 *(232)* used the same strain and all rats ingested phytoestrogen-containing diet (600 µg/g). At PND 80 one half of the animals on the phytoestrogen diet were switched to phytoestrogen-free food on PN 80 then all rats were killed on PN 120. The AVPV was examined by Nissl stain and the expected sex difference (female>male) was only noted in the animals that remained on phytoestrogen-containing chow. Notably males moved to phytoestrogen-free chow had larger AVPV volumes

than the control males that remained on phytoestrogen-containing diet. This demonstrates that the neuronal changes can occur in the adult brain in the AVPV when phytoestrogens are eliminated from diet, but this only occurred in males. Finally another sexually dimorphic region, the SDN, was examined in another strain, Sprague Dawley rats by Taylor *et al.*, 1999 *(724)*. Dams were placed on phytoestrogen-free or phytoestrogen-containing (200 μg/g) chow when mated and fetuses were taken on E20. Western blots were used to quantify calbindin in the medial hypothalamus (most of the calbindin in this region would be in the SDN). The normal sex difference (male>female) was only noted in fetuses from the phytoestrogen consuming dams. On the phytoestrogen-free diet, females had significantly more calbindin protein than the phytoestrogen-ingesting females. Neither of these sex differences is related to the expression of anxiety. Interestingly, both of these classically described neural differences require phytoestrogens in chow.

3.7 Conclusions

- Evidence is sufficient to conclude that genistein, produces developmental toxicity in male and female mice and rats. The panel considered oral or parenteral exposure between birth and weaning at dose levels (between 37.5 and 50 mg/kg body weight/day in mice; between 25 and 100 ppm in rats) that produce blood levels similar to those reported in children. In female mice dosed via the oral or parenteral routes, adverse effects were manifested as histomorphological changes of the reproductive tract and the mammary gland, a decrease in fertility, and/or a decrease in numbers of live pups per litter. In a multigenerational study in rats with dietary exposure to genistein, changes were observed in mammary gland morphology in both sexes (25 ppm males and 100 ppm females). While not a definitive marker of developmental toxicity, consistent changes in the age of vaginal opening were seen in mice and rats. Some biological changes in a discrete brain region in female rats have been documented at 40 mg/kg bw/day. The experimental animal data are considered relevant to the assessment of human risk.
- Evidence for daidzein, equol, and glycitein is insufficient due to a paucity of relevant studies. However, relative estrogen receptor binding activity of equol and daidzein are broadly similar to genistein.
- Evidence is insufficient to conclude that soy infant formula or other soy exposures, including soy-based diets, produces or does not produce developmental toxicity in experimental animals. While a few studies have examined the developmental effects of soy infant formula or other soy exposures and some have identified potential developmental effects, these studies have yet to be replicated. The experimental animal data are considered relevant to the assessment of human risk.
- Evidence is insufficient to conclude that soy infant formula produces or does not produce toxicity with infant exposure in girls or boys at recommended intakes manifested by the following endpoints: bone mineral density, gastrointestinal effects, allergy/immunology, thyroid function, reproductive endpoints, cholesterol, diabetes mellitus, and cognitive function.
- Evidence is sufficient to conclude that use of soy infant formula in healthy full-term infants does not impair growth during infancy. This conclusion is based upon a large number of studies of small sample size that consistently show similar growth trajectories of anthropometric measurements.

4.0 SUMMARY

4.1 Summary of Human Exposure

Infant exposure to isoflavones occurs primarily through the consumption of soy formula. The isoflavones typically found in soy formula are genistein (58–67%), daidzein (29–34%), and glycitein (5–8%) with the majority of isoflavones occurring as glycosides. Total isoflavone levels found in soy formula worldwide was 10–47 mg/L formula ("ready-to-feed" equivalent). These levels are two orders of magnitude higher than those observed in casein-based formula or breast milk.

The degree to which infants are exclusively fed soy formula *versus* a combination of soy and non-soy formula and/or breast milk is not clearly known. It is estimated that upwards of 25% of newborns or infants in the US are fed soy formula at some point. Exposure to soy formula also varies depending on developmental stage (*e.g.*, weaning), and cultural variations in soy formula and soy product usage are known to exist. Prenatal exposure to soy isoflavones *via* maternal soy intake is also possible.

Recent sales of soy formula in the US represent ~12% of the total dollar sales for infant formula (personal communication with Robert Rankin, Manager of Regulatory and Technical Affairs at the IFC, October 13, 2009). US sales data also suggest a 50% reduction in soy formula use over the last decade (public comment from the International Formula Council (IFC), received December 3, 2009 and personal communication with Dr. Haley Curtis Stevens, IFC). When sales are considered as a surrogate measure of actual reported usage, these data provide a lower indication of usage compared to other frequently cited estimates. The usage and sales of soy formula also varies geographically ranging from 2 to 7% of infant formula sales in the UK, Italy, and France , 13% in New Zealand *(35; 36)*, to 31.5% in Israel *(37)*.

In the US, total isoflavone intake by infants was estimated at 2.3–9.3 mg/kg bw/day, depending on age of the infant. The estimated intake for genistein, expressed in aglycone equivalents, ranges from 1.3 to 6.2 mg/kg bw/day. These intakes are several orders of magnitude greater than those infants who consume breast milk or a cow's milk-based formula (**Table 27**). Soy formula fed infants have higher daily intakes of genistein and other isoflavones compared to other populations (excluding regular consumers of soy supplements) (**Table 28**).

Guidelines regarding the use of soy formula have been issued by both the American Academy of Pediatrics (AAP) *(42)* , the European Society for Paediatric Gastroenterology Hepatology and Nutrition (ESPGHAN) Committee on Nutrition *(36)*, and reviewed in the literature *(35; 43; 44)*. In general the exclusive use of soy formula over non-soy and/or breast milk is not recommended. The only real indications for use are instances where the family prefers a vegetarian diet or for the management of infants with galactosemia or primary lactase deficiency (rare). Soy formula is not currently recommended for preterm infants.

Mean blood-based levels of isoflavones in infants fed soy formulas are considerably higher than other populations, including vegans and Japanese adults (Table 29; reproduced below as **Table 158**). For example, concentrations of total genistein in whole blood samples from US infants fed soy formula are 1455 ng/ml at the 75th percentile, range of 13.5 to 3562.9 ng/ml *(94)* (personal communication with Dr. Yang Cao, NIEHS).

Table 158. Comparison of Blood-Based Levels of Genistein and Daidzein in Infants Fed Soy Formula to Other Populations

Population	*n*	*Sample*	*Average Total Isoflavone Concentration, nM (ng/ml)*		
			Genistein	*Daidzein*	*Reference*
US infants (soy formula)	27	Whole blood (spots)	2801 **(757)**, geometric mean 3296 **(891)**, median 5385 **(1455)**, 75th percentile	1007 **(256)**, geometric mean 1081 **(275)**, median 2040 **(519)**, 75th percentile	Cao et al., 2009 *(94)*
US infants (soy formula)	7	Plasma	2531 **(684)**	1160 **(295)**	Setchell et al., 1997 *(84)*
US infants (cow milk formula)	30	Whole blood (spots)	52.5 **(14.2)**, geometric mean	**(5.5)**, geometric mean	Cao et al., 2009 *(94)*
US infants (cow milk formula)	7	Plasma	11.7 **(3.16)-**	8.1 **(2.06)**	Setchell et al., 1997 *(84)*
US infants (breastfed)	20	Whole blood (spots)	40 **(10.8)**, geometric mean	20.8 **(5.3)**, geometric mean	Cao et al., 2009 *(94)*
US infants (breastfed)	7	Plasma	10.3 **(2.77)**	5.9 **(1.49)**	Setchell et al., 1997 *(84)*
US adults, Omnivorous	209	Serum	17.4 **(4.7)** < LOD–**(203)**, range	15.3 **(3.9)** < LOD–**(162)**, range	Valentin-Blasini et al., 2003 *(107)*
Finland, Vegetarians	14	Plasma	17.1 **(4.6)**	18.5 **(4.7)**	Adlercreutz et al., 1994 *(109)*
Oxford, UK Vegans and vegetarians	70	Plasma	148 **(40)**	78.7 **(20)**	Peeters et al., 2007 *(113)*
Japanese women at delivery	51[a] or 194[b]	Serum	26.6 **(7.2)**[a] – 116.5 **(31.5)**[b]	7.1 **(1.8)**[a] – 50.2 **(12.8)**[b]	[a] Todaka et al., 2005 *(103)*; [b] Nagata et al., 2006 *(102)*
Japanese cord serum at delivery	51[a] or 194[b]	Serum	71.8 **(19.4)**[a] – 126.9 **(34.3)**[b]	16.9 **(4.3)**[a] – 38.6 **(9.81)**[b]	[a] Todaka et al., 2005 *(103)*; [b] Nagata et al., 2006 *(102)*
Japanese men (traditional diet)	6	Plasma	90.4**(24)** – 1204 **(325)**, range	58.3 **(15)** – 924 **(235)**, range	Adlercreutz et al., 1994 *(109)*

The geometric mean value of total genistein in the infants fed soy infant formula (757 ng/ml) was 53.3- and 70.1- times higher compared to levels measured in infants fed cow milk formula (14.2 ng/ml) or breastmilk (10.8 ng/ml), respectively. The maximum obtained value in the Cao *et al.*, 2009 study is approximately 11 times higher than the maximum genistein concentration (325 ng/ml) detected in plasma in a small study of Japanese men, n=6 *(109)*. Average blood levels of total genistein in the soy formula-fed infants (757 ng/ml) are ~160-times higher than the mean levels of total genistein in omnivorous adults in the US (4.7 ng/ml) reported by Valentin-Blasini *et al.*, 2003 *(107)*.

4.2 Summary and Conclusions of Pharmacokinetics

There are no pharmacokinetic data for individual isoflavones following administration of soy-based infant formula to infants or children. Thus, reliable estimates of exposure, as defined by area under the plasma concentration-time curve (AUC), are not available and therefore preclude meaningful comparisons of exposure between infants receiving recommended intakes on normal feeding schedules and experimental animal models. There are currently no estimates of variability in exposure to individual isoflavones (genistein, daidzein, equol and glycitein), or pharmacokinetic parameters describing the disposition of those isoflavones, following recommended intakes of soy-based infant formula. Given the heterogeneity of the human infant population, population studies incorporating measures of systemic exposure (i.e., accurately timed plasma samples and quantitative urinary recoveries) are essential to identify a potentially susceptible subgroup, if one exists.

Nevertheless, human data demonstrate that hydrolysis of genistin, the glycoside conjugate of genistein that is present in soy formula, occurs in both adults and infants such that systemic exposure to genistein occurs *(84; 94; 142)*. The mechanism of deconjugation may involve bacterial hydrolysis in the gastrointestinal tract, although some data suggest that the glycoside conjugate may be absorbed. Irrespective of the mechanism, systemic exposure to genistein has been documented in soy formula-fed infants, with total genistein concentrations (isoflavone and conjugates) ranging from 13.5 to 3562.9 ng/ml *(94)* (personal communication with Dr. Yang Cao, NIEHS). Although it is generally accepted that the majority of isoflavone readily available in accessible body fluids (*e.g.*, blood and urine) is in a conjugated form, the relative proportion of measurable isoflavone that is available as aglycone, glucuronide or sulfate has not been determined for infants or children fed soy-based formula. Thus, the age-related differences in glucuronidation and sulfation of individual isoflavones are unknown, and comparison of the relative exposure to unconjugated forms of isoflavones between infants and adults is not possible.

Equol exposure in human infants following daidzein intake is relatively low compared to animals of a comparable developmental stage. This observation is relevant for the risk assessment of daidzein, but not of importance to the risk assessment of soy formula due to the detection of equol in infants independent of feeding type.

Genistein, daidzein, and equol all have weak affinity for estrogen receptors (ERs), as compared to an endogenous agonist such as estradiol, and the ability to activate ER-dependent transcription. There is also evidence suggesting that many of the developmental and reproductive endpoints observed in experimental models could be mediated through ER-dependent mechanisms given the central role of ERs in modulating these systems and the similarity of effects observed as compared to those found in response to estradiol. However, there are limited studies to date that have definitively established the

essential role of ERs in relevant toxic endpoints (e.g., insufficient studies using null mice, siRNAs, etc.). One study has demonstrated that ERβ is required for genistein-induced multioocyte follicles in C57BL/6 mice, as this effect is not found in genistein-treated ERβ-null mice *(591)*. However, this study also demonstrated that inhibition of tyrosine kinase activity by genistein could also contribute to the mechanisms of other effects resulting from genistein exposure in this model. This finding is consistent with gene expression profiling studies showing that the signaling mechanisms induced by selective ER agonists are substantively different than those resulting from treatment with isoflavones. This suggests that other receptor-dependent and/or receptor-independent (e.g., epigenetic) mechanisms could contribute to the etiology of isoflavone-induced toxicities.

4.3 Summary and Conclusions of Developmental Hazards

4.3.1 Humans

With the exception of conclusions for growth (discussed below), the overall evidence was considered insufficient to reach a conclusion on whether use of soy infant formula produces or does not produce developmental toxicity with infant exposure in girls or boys at recommended intake levels. Commonly encountered limitations included the non-random or unspecified method of assignment to feeding groups, the use of self-selected breast- and formula-feeding mothers, failure to control for the reasons for which soy formula was used, and the early and inconsistent introduction of solid foods. Studies that compared outcomes in children randomized to soy or cow-milk formula were considered the most reliable, particularly when parents and outcome assessors were masked to formula assignment. The studies also were evaluated based upon adequate sample size by gender and feeding group, presence or absence of longitudinal follow-up, validation of exposure to soy formula, and appropriate adjustment for potential confounding variables. The evidence was considered sufficient to conclude that use of soy infant formula in healthy full-term infants does not impair growth during infancy. This conclusion is based upon a large number of studies of small sample size that consistently show similar growth trajectories of anthropometric measurements.

The expert panel had several remarks related to the quality of the available information for specific endpoints:

- Full-term healthy infants fed soy formula have comparable growth as compared to full-term, healthy infants fed breast-milk or cow-milk formula. Soy formula feeding may not support the growth of premature infants and causes an increased incidence of rickets. Since the AAP does not recommend the use of soy formula for premature infants, current literature on the subject is scant.
- Soy infant formula may or may not cause reproductive toxicity in boys and girls based on current evidence. Preliminary data addressed in the panel's report do not allow firm conclusions on this effect. Limited retrospective data suggest that soy formula fed infants may demonstrate premature thelarche (the start of breast development at the beginning of puberty) *(576)*. The only other study considered to have limited utility that included reproductive parameters did not have sufficient power to rule out increased risks *(32)*.
- Soy infant formula may or may not cause adverse effects on thyroid function in male or female infants and children. A special cohort of infants and children with congenital hypothyroidism (CH) fed soy infant formula demonstrated a delay of TSH levels to return to normal after adequate treatment; these children may need increased doses of levothyroxine and closer

follow-up. However, the studies that specifically targeted infants and children with CH were case-studies, which results in limited inferences.

- All studies of gastrointestinal effects reviewed for this report were classified as having no utility. However, extensive reviews by AAP and ESPGHAN have reported adverse effects in a subset of infants with documented cow milk protein allergy; infants with documented cow milk protein-induced enteropathy or enterocolitis frequently are as sensitive to soy protein and should not be given soy protein formulas.
- Sensitivity to soy protein during human infancy and childhood may occur, but, it appears to affect a very small subset of the population. Villous atrophy was reported with the use of soy formulas, but these inferences were of no utility due to the study designs being case reports.
- One cholesterol study was reviewed for this report *(199)* and it was classified as having limited utility. The fractional cholesterol synthesis rates were significantly greater in infants fed soy formula (12.02 %/day) as compared to human breast milk fed infants (2.97 %/day). This rate for soy formula fed infants was not statistically significantly different when compared to cow-milk fed infants. These findings suggest that infants respond to different dietary cholesterol intakes through altered cholesterol synthesis rates.

One diabetes study was reviewed for this report *(581)*. The design of the study was a retrospective case-control, which examined feeding histories of diabetic children versus controls to study the effect of breast feeding on the development of diabetes. Retrospective collection of infant feeding information on average of 14 years was used to classify feeding groups. As a result of this type of classification, twice as many diabetic children had been said to have been fed soy formulas compared to control. Poor recall is also associated with the lack of information regarding the duration and quantity of soy formula feeding. Furthermore, soy formula exposure was said to have been mixed with cow milk formula exposure. No association between diabetes and exposure to soy formula was found. As a result of these deficiencies, the study was classified as no utility.

Two cognitive function studies were reviewed for this report *(582; 583)* and were considered of no utility. One study compared cognitive performance in children who were fed soy formula or human breast milk. After adjustment of confounding variables, no differences were found in the outcome measures. An additional factor which makes this study of no utility is the possibility of recall bias (9-10 year span). The second study compared soy formula fed infants to cow milk formula fed infants. Spectral EEG was used to assess brain development. Duration of soy feeding was one month. None of the spectral variables differed between the exposure groups. The duration of feeding for one month may not have been sufficient to discern differences in EEG patterns.

4.3.2 Experimental Animals

While experiments designed to provide information of potential health risks in humans are ideally conducted in the same species, there are numerous reasons why this is often not possible or practical. The purist might then conclude that we cannot do any relevant research. However, there are numerous examples of both translational uses of animal studies to develop targeted treatments and also of pure basic science work to inform human studies. Animal models are considered to be useful in hazard identification since they allow investigators to control for potential confounders and conditions of exposure that cannot be achieved in epidemiology or clinical studies and thus causal associations and mechanistic pathways can be addressed. Estrogen receptors are expressed in key target tissues,

regulate estrogen function in a similar manner in both rodents and humans, and oral administration of phytoestrogens results in similar circulating levels in both rodents and humans.

Animal studies have been used for risk assessment and for the development of new hypotheses about mechanisms of action. Experiments in mice, rats, pigs and non-human primates are routinely used to assess effects of various compounds on endpoints including but not limited to: reproduction, neural development, behavior, and toxicity. In the case of soy and isoflavone studies, routes of exposure have included oral and subcutaneous administration. The expert panel tried to preferentially evaluate studies that used oral administration since it best mimics the human route of exposure. However sc injection studies were considered when they used dose levelswhich produced blood levels representative of those measured following human exposure were also considered. Most weight in the assessment was given to those studies that exposed developing animals to soy, or in most cases the specific isoflavone genistein, solely during the period from birth to weaning. During this window of exposure, which approximates the timing of soy formula ingestion in infants, genistein can affect the following in developing animals: puberty onset, neural differentiation, reproductive tract morphology, and mammary gland organization.

While the porcine model was seen to have important advantages over rodents as a model of human exposure because they do not produce equol from daidzein, the panel found that there was a paucity of studies using the porcine model. Furthermore, although human infants do not produce comparably high quantities of equol, they are exposed to daidzein which has a similar affinity for the estrogen receptors. While it is recognized that rodents do not produce SHBG, they produce other sex hormone binding proteins. Pharmacokinetic issues were considered by the panel, and key oral and subcutaneous injection studies were shown to produce similar blood concentrations and circulating patterns *(216; 217)*. Taken together these results suggest that the subcutaneous route of administration can be used for hazard identification studies. The panel notes that all of the animal models include exposure to genistein.

4.4 Overall Conclusions

The Expert Panel has minimal concern for adverse developmental effects in infants fed soy infant formula.

This conclusion is based on:

- Lack of clarity on whether studies in experimental animals treated with genistein only can be extrapolated to infants fed soy infant formula, i.e., exposure to a single isoflavone versus soy infant formula
- Interpretation of findings from experimental animals as demonstrating adverse effects, i.e., advanced vaginal opening, effects on the mammary gland in the context of interspecies comparisons
- Although there are a large number of experimental animal studies published on genistein or soy, there are only a limited number of studies where experimental animals were treated only during the relevant life stage of birth to weaning. Multigenerational studies do not permit discerning effects attributed to gestational or lactational exposure.
- However, a number of studies in experimental animals and one study in humans reported effects related to the reproductive system and this elevates the concern from "negligible" to "minimal."
- Studies of sufficient quality in humans have not been conducted to address the concerns raised from the experimental animal findings or to identify previously unrecognized endpoints.

The panel were in favor of the conclusion by a tally of 10 yes and 2 no votes.[16]

Dissenting opinions:
One committee member, Dr. Jatinder Bhatia, expressed negligible concern for adverse developmental effects in infants fed soy infant formula based on a lack of finding in humans and lack of experimental data in animals fed soy formula.

One committee member, Dr. Ruth Etzel, expressed some concern for adverse developmental effects in infants fed soy infant formula. Dr. Etzel supported her level of concern conclusion based on the following three points: 1) There are sufficient signals of adverse reproductive and developmental effects in experimental animals to worry about long term effects on development of infants; 2) Infants are exquisitely sensitive to the effects of exogenous chemicals during early life, and naturally occurring genistein in soy formula has a much stronger estrogenic effect than non-natural estrogenic compounds on the developing reproductive system; and 3) the absence of evidence of an effect in human studies is not the same as evidence of absence of an effect, particularly given the paucity of human data to inform the conclusion.

4.5 Critical Data Gaps and Research Needs

4.5.1 Pharmacokinetics

Knowledge deficits and research needs can be summarized as follows:

1. Available pharmacokinetic data in human infants essentially are equivalent to biomonitoring data, and permit crude contextual assessment of the animal data. Full pharmacokinetic data are needed in infants fed soy formula to inform the questions of interest.

2. Although plasma/blood data approximating steady state have been collected, the temporal relationship with feeding is unknown. In the absence of full pharmacokinetic profiles, accurate timing of blood/plasma sampling relative to feeding would be an improvement over the available data.

3. From a purely scientific perspective, pharmacokinetic data for a range of doses with individual isoflavones would generate the most interpretable data with respect to understanding the dose-response relationship between isoflavones and reproductive outcomes. However, this is unlikely to ever be practical or ethically possible as isoflavones are administered to the population of interest, infants, as a complex biological mixture. Human ethics committees may be reluctant to approve investigations involving compounds of uncertain toxic potential, and parents are unlikely to consent to their infants participating in such a study. Pharmacokinetic studies following administration of soy-based formulas may be more acceptable to both human ethics committees and parents.

4. The adult data indicate that glucuronidation and sulfation are the two primary pathways for isoflavone biotransformation. However, high quality quantitative assessment of their relative importance is not available, including the extent of variability of glucuronidation and sulfation in relevant populations.

[16] The expert panel had 14 members but the Chair, Dr. Gail McCarver, does not vote and Dr. Michael Rybak was not present on the day of the vote.

5. *In vitro* data on the enzymes most important for isoflavone disposition in humans are limited. Furthermore, the tissue distribution of those isoforms (especially in liver and intestine), and the relative level of expression of individual isoforms in target tissues is unknown. This knowledge deficit precludes further investigations of ontogeny as well as pharmacokinetic modeling studies.

6. Studies to firmly establish whether ERs are, or are not, required for isoflavone-dependent toxicity are needed.

7. Studies to determine the relative role of epigenetic mechanisms that may or may not be required for isoflavone-dependent toxicity are needed.

4.5.2 Human Epidemiological

1. Based upon the current state of the science, a basic (but difficult) question to address is, "If ingestion of soy formula results in altered onset of puberty, and/or alteration of reproductive organs, then what would the cumulative exposure of soy formula have to be in order to observe such effects?"

2. Larger (in terms of sample size) and longer (time span) longitudinal, prospective cohort studies are needed, e.g., a longitudinal study that captures soy exposure from birth through puberty. This is likely to be quite challenging due to current indications for soy formula and declining prevalence of use. One opportunity would be to collect appropriate measures of soy protein exposure in the National Children's Study

3. Human studies should include endpoints that have been used in previous studies (growth, bone mineral density, and cognitive performance), in addition to reproductive endpoints that reflect the greater time span of the study, such as onset of puberty. The first endpoints in the series collected should reflect a "pure" exposure, i.e., minimal cross-feeding with breastmilk or cows milk-based formula.

4. Case-control studies should include longer term endpoints such as breast cancer. Again, longer term endpoints could be quite challenging due to the dietary drift that occurs in the short term that will almost certainly occur in the long-term.

5. Continued observational studies of thyroid function in infants fed soy infant formulas are needed.

6. Studies that combine clinical outcomes with objective measures of soy protein exposure are needed.

4.5.3 Experimental Animal

While animal studies are essential to hazard identification and to address potential mechanisms of action, numerous data gaps were identified in this assessment.

1. There is a paucity of studies that examined the effect of soy formula as opposed to individual compounds. Use of full foods is thought to account for potential interactions amongst chemical constituents of the formula and thus is more representative of human exposure than studies employing single soy phytoestrogens.

2. There are scant studies that examined the effects of isoflavones other than genistein.

3. While rodents are widely used in hazard identification there are important limitations to rats and mice as models for human exposure. Differences in comparative physiology can have important implications to the model selection and the relevance of the data generated. Of note, rodents unlike human infants produce significant amounts of equol from daidzein. The total estrogenicity of soy needs to be more carefully considered in future studies.
4. The effects of soy need to be examined in the most relevant period of development.
5. Activities other than estrogenicity of soy products need to be evaluated at exposure concentrations representative of human exposure.
6. The impact of species differences in bioavailability of sex steroid and isoflavones may influence the potency of soy in rodent models and requires further investigation.
7. There is limited information concerning the effect of early life exposures to soy proteins and isoflavones on animal susceptibility to subsequent chemical insults in later life. This question addresses the concern that early life exposure to isoflavones or soy proteins may have a programming effect that alters risk for hormonally dependent diseases such as breast cancer.
8. More studies examining the effects of soy or isoflavone exposure during infancy on developmental effects on puberty are needed. The relevance of some developmental effects to human health, such as vaginal opening (time of puberty onset) in rodents, is uncertain. More studies, including additional measures of puberty (e.g., ovulation, gonadotropin secretion and onset of estrus cyclicity) in rodents and human studies on timing of sexual maturation, are needed.
9. The long term effects of soy or isoflavones during infancy should be addressed in animal and human studies of fertility, reproductive senescence (menopause), and life span.
10. Animal and human studies that assess effects of soy or isoflavones from birth to weaning on non-reproductive behaviors are needed.

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APPENDIX III: PUBLIC COMMENTS AND PEER REVIEW REPORT

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Public comments received during the NTP-CERHR evaluation of soy infant formula and the peer review report for the draft NTP Brief on soy infant formula are available on the NTP website at *http://ntp.niehs.nih.gov/go/36487*.