1. BACKGROUND AND PURPOSE OF THE COMMENTS

On 9 June 2009, the Agency received a formal request from the Directorate General for Health (DGS) for a health risk assessment (HRA) of exposure to category 3 \(^1\) (R3) reprotoxic (according to Directive 67/548/EEC\(^2\)) and/or endocrine disrupting (ED) substances found in consumer products marketed in France. This expert appraisal covered the general population, including vulnerable populations and people in the workplace handling so-called ‘consumer’ products in the context of their professional activities (excluding manufacture, processing, distribution and disposal).

In this context, in 2013, ANSES published an Opinion "on the assessment of the risks associated with bisphenol A for human health, and on toxicological data and data on the use of bisphenols S, F, M, B, AP, AF, and BADGE" regarding the risks to human health related to bisphenol A (BPA) taking into account not only exposure related to consumer goods but also exposure related to other media (drinking water, food, house dust, air). This Opinion presented the expert appraisal work undertaken by a Working Group on Endocrine disruptors and category 3 reprotoxic substances (ED WG) set up by ANSES in 2010. The expert assessment report on the health effects of BPA produced by the ED WG was submitted to several expert groups at ANSES and validated by the Expert Committee on Assessment of the risks related to chemical substances in February 2013 (ANSES, 2013).

In the ANSES expert report published in March 2013 on the risks to human health related to BPA, the experts recommended monitoring the results of the work undertaken under the supervision of the US National Toxicology Program (NTP), with funding from a joint Food and Drug Administration/National Institute of Environmental Health Sciences (US FDA/NIEHS) programme, in order to revise the conclusions of the ANSES expert report where appropriate. The article published by Delclos et al. in February 2014 entitled "Toxicity evaluation of Bisphenol A administered by gavage to Sprague Dawley rats from gestation day 6 through postnatal day 90" briefly summarises the results of the US FDA/NCTR 2013 study.

The US FDA/NCTR 2013 study was a large-scale animal study on continuous exposure to BPA from gestation day 6 (GD6) to postnatal day 90 (PND 90), when a number of animals were

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\(^1\) Substances classified as category 3 reprotoxic according to Directive 67/548/EEC are now classified as toxic to reproduction, category 2 according to (EC) Regulation no. 1272/2008, known as the CLP (Classification, Labelling, Packaging) Regulation. In this document, substances are classified based on the CLP Regulation.

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Euthanised and tissues were collected. It complied with the criteria of Good Laboratory Practice (GLP) and was conducted in accordance with the specific guidelines of the NTP. The study included a wide range of tested doses (2.5 – 8 – 25 – 80 – 260 – 840 – 2,700 – 100,000 – 300,000 µg BPA/kg bw/day) administered by gavage using a stomach tube (mothers or pups after 5 days) or orally for newborns from the first day after birth. This descriptive study relied primarily on the observation of overall morphological-histological and biochemical criteria. Two positive control groups for the characterisation of oestrogenic effects were included in the study: treatment with 0.5 and 50 µg ethinyl oestradiol (EE2) per kg bw/day. Two negative control groups were also included (vehicle\(^3\) and without treatment). ANSES decided to initiate an initial request to analyse this study, in particular to assess whether the new data of the US FDA/NCTR 2013 study were likely to influence the conclusions of the ANSES collective expert appraisal of 2013. The data of Churchwell et al., 2014 supplement the US FDA/NCTR 2013 study as regarding the toxicokinetics of BPA. Following the evaluation by the expert rapporteurs appointed by ANSES, an opinion was released by ANSES the 7 August, 2015.

In the European regulatory field, ANSES submitted in 2013 a dossier to the European Chemicals Agency (ECHA) in the context of the Classification, Labelling and Packaging (CLP) Regulation on the classification of BPA as a “reprotoxicant category 1B” which was adopted in March 2014 (see ECHA website\(^4\)). In 2014, in the context of the REACH regulation, ANSES sent a restriction proposal to ECHA to limit the use of BPA in thermal paper, based ANSES 2013 BPA risk assessment report. The restriction decision was published in the EC official journal in 2016 (https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX:32016R2235). In June 2017, the Member States Committee at ECHA, on the basis of ANSES contribution, agreed with France to identify BPA as a “Substance of very high concern” according to REACH article 57f provisions with regard to its endocrine properties for Human health (ECHA, 2017).

The preliminary NTP CLARITY-BPA Core study report presents the results of the core, guideline-compliant, chronic, extended-dose-range study of bisphenol A (BPA) in rats conducted as part of the CLARITY–BPA Research Program. The U.S. Food and Drug Administration’s National Center for Toxicological Research (NCTR) conducted the study under the auspices of the National Toxicology Program and prepared the draft report in collaboration with the National Institute of Environmental Health Sciences (NIEHS). The draft NTP Research Report was made available to the public by February 23, 2018, at https://ntp.niehs.nih.gov/go/rrprp Submission of written comments is welcome until April 12, 2018. The peer-review meeting will be held at NIEHS in Research Triangle Park, NC and is open to the public. This meeting is tentatively scheduled for April 26, 2018.

2. ORGANISATION OF THE EXPERT APPRAISAL

ANSES entrusted the expert appraisal to several expert rapporteurs in the Working Group on Endocrine disruptors and category 3 reprotoxic substances (ED WG) with toxicological expertise, in particular experts on the mammary gland, male and female reproductive systems, thyroid and metabolic diseases. The methodological and scientific aspects of the work were presented to the ED WG on April 9, 2018. Each expert was mandated to assess a specific part of the preliminary

\(^3\) Vehicle control corresponding to the negative control group that received the vehicle or diluent.

\(^4\)https://echa.europa.eu/fr/harmonised-classification-and-labelling-previous-consultations/-/substance-rev/3364/term?_viewsubstances_WAR_echarevsubstanceportlet_SEARCH_CRITERIA_EC_NUMBER=201-245-
&_viewsubstances_WAR_echarevsubstanceportlet_DISS=true
NTP CLARITY-BPA Core study namely on the mammary gland, the male and female reproductive system and on metabolism and obesity

ANSES analyses interests declared by experts before they were appointed and throughout their work in order to prevent risks of conflicts of interest in relation to the points addressed in expert appraisals.

The experts' declarations of interests are made public on ANSES's website (www.anses.fr).

The results of the expert appraisal presented below take into account the experts' comments. They cover specific points of the preliminary NTP CLARITY-BPA Core study. Thus, the issues raised in the context of this expert appraisal are as follows.

3. COMMENTS OF THE WORKING GROUP ON ENDOCRINE DISRUPTORS

This study is unique in that it characterises the effects of BPA following a pre-natal exposure and a post-natal exposure until PND 21 followed by a continuous dosing to sacrifice at 1 year or 2 year (CD for continuous dosing) or without further treatment until sacrifice at 1 or 2 years (SD for stop dosing). The study protocol was rather complex and combined a daily dosing of the dam from GD 6 until parturition with BPA, EE2 (for continuous exposure only) or vehicle and a post-natal treatment until PND 21 with one animal per sex per litter assigned to the following arms:
   1) Continuous dosing until sacrifice at 2 years; (CD, Terminal)
   2) Continuous dosing until sacrifice at 1 year; (CD, Interim)
   3) No further treatment after PND 21 until sacrifice at 2 years; (SD, Terminal)
   4) No further treatment after PND 21 until sacrifice at 1 year, (SD, Interim).

The main characteristics of the study protocol were as follows:
- Oral gavage from GD 6 and then directly to pups from PND 1 until termination at 1 or 2 years of age for the continuous study or only until PND 21 with sacrifice at 1 or 2 years of age.
- Sprague-Dawley rats
- BPA doses of 2.5; 25; 250; 2,500; 25,000 µg/bw/day
- Vehicle: 0.3% carboxymethylcellulose (CMC)
- Administration with a modified Hamilton Microlab ML511C programmable IL5V pumps
- Diet: casein diet 10 IF irradiated 5K96 (Suppl App XII) low in phytoestrogens
- Contamination of BPA in diet: 0.05 µg/bw/day (Suppl App XIV)

Other characteristics for the two protocols:
- Random repartition of female rats after stratification by body weight so that the mean body weight per group is identical among groups (Suppl app VI)
- For the parental generation (F0), 600 males and 600 females at weaning (PND 21) were obtained from the NTCR breeding colony in 5 groups of 120 each. Mating with animals of 10-15 weeks was conducted in 5 separate cohorts (see p44) spaced 4 weeks apart (see Table 3). Animals were housed in polycarbonate cages and watered using polycarbonate bottles until inclusion in the experiment.
- Females were individually housed upon evidences of mating
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- Feed consumption measured weekly from the start of dosing for approximately the next 13 weeks and monthly afterward.
- At weaning, one pup per sex per litter was assigned to the following arms:
  o Continuous dosing to sacrifice at 2 years (46/50/sex/BPA dose and 26/sex/EE2 dose)
  o Continuous dosing to sacrifice at 1 year (20-26/sex/group)
  o No further treatment after PND 21 until sacrifice at 2 years (46-50/sex/BPA dose)
  o No further treatment after PND 21 until sacrifice at 1 year (20-26/sex/BPA dose)

Phenotype:
For the one-year interim sacrifice, food but not water was removed from cages on the evening before the scheduled necropsy. Animals assigned to the two-year sacrifice were not fasted, and no clinical chemistry, hematology, organ weights, or sperm evaluations were conducted for these animals.

Body weight, litter parameters, age at vaginal opening, vaginal cytology, clinical chemistry (at sacrifice), sperm parameters (at sacrifice), organ weights (at sacrifice), histopathology (interim and terminal at sacrifice).

Regarding metabolism: Body weight, glycaemia, ALAT, ASAT, γGTP, total protein, albumin, cholesterol, triglycerides, blood urea nitrogen, creatinin, total bile acids, leptin, insulin, T3, T4 and TSH.

A dose trend analysis was performed on BPA-treated groups versus vehicle, followed by Pair-wise comparisons comparing individually the five BPA-dose groups to the vehicle control group (similarly performed for the EE2-dose groups).

To be noticed that Ethinyl Estradiol (EE2) doses of 0.05 and 0.5 μg/bw/day were included in the terminal and interim sacrifice continuous dose arm resulting in an average of 10 pg/ml (30 ppm) at adult age. No positive control group was included in the stop dose study.

Incidence rate of neoplastic and non-neoplastic lesions was determined after immuno-histochemical and/or histopathological examination of each tissue in all animals including those that died before the scheduled sacrifice. Statistical analysis was reported for all diagnosed lesions but only analyses of the most representative neoplastic and non-neoplastic lesions were presented for females and males in the body of the report. The poly-3 statistical test applied for the analysis of the 2-year pathology data was adjusted for mortality.

Methodological comments:

Some methodological information is needed such as:
- The number of histopathological slides/organ. This is particularly needed for the female reproductive organ analysis as well as for the mammary gland examination;
- Definition or nomenclature of the reported histopathological lesions.
- Microscope views illustrating the histopathological lesions.

Information on the hormonal status of the animals (other than just thyroid) is mandatory to enable a proper analysis of the endocrine parameters. In particular, the estral stage at which females, especially at 1 old of age, were sacrificed should be indicated. Similarly, the time of each sampling should be indicated to enable sampling time to be considered as a confounding parameter.
F0 breeders were maintained with their dams under the standard conditions used in the NCTR colony and namely housed in polycarbonate cages and given water from polycarbonate bottles. These conditions may have led to BPA exposure and it is not discussed how it has been taken into account in the study. Once assigned to the study at weaning, the F0 breeders were then housed in polysulfone cages and given water from glass water bottles with silicone stoppers. As F0 breeders housing conditions may lead to expose constantly the dams to BPA, it is of concern to precise how this continuous BPA exposure will be taken into account.

Based on the BPA solubility characteristics and the wide range of BPA tested dose levels (from 2.5 to 25,000 µg/bw/day), this leads to administration of dosing suspension or solution of BPA in CMC. Did the NTP team investigate the impact of this difference of formulation in terms of systemic bioavailability?

In order to preclude BPA or EE2 cross contamination between rodent cages, were any devices being put in place between cage racks?

Due to size of this core study, NCTR breeding colonies were assigned to 5 different cohorts of 120 each with different mating periods (10-15 weeks of age). 4 weeks apart was conducted in 5 separate cohorts (see report on p44 and Table 3). These assignments could be seen as 5 independent studies. For example, although their effects are low in rodent laboratories, seasonal changes may impact the reproductive behaviour of rodents, there could be an impact on the laboratory technician assigned... Therefore, the relevance to pool results from 5 separate cohorts spaced 4 weeks apart may be questioned. We question the relevance of the statistical analysis performed and would like more explanation about the model used, and the rationale for pooling the 5 cohorts.

**Specific comments**

*Results obtained with the positive control EE2.*

The absence of EE2 impact on endometrium hyperplasia (at least at the highest dose level of 0.5 µg/bw/day), vaginal opening timing, sperm parameters and mammary gland (in male) may call for further information. Therefore, to better capture this new data set, the expected effects of the EE2 positive control for female reproductive system, mammary gland, metabolism and obesity, male reproductive system including prostate and cardiovascular system should be substantiated in the final report. Indeed, when expected results from exposure to a positive control are not reproduced, it questions the validity of the entire study.

*Results obtained within control groups.*

Non-neoplastic lesions were observed with incidence as high as 60% in animals continuously exposed to vehicle, these effects were for example endometrium cystic hyperplasia at terminal sacrifice. These type of results needs to be discussed in the final report. Most importantly, there should be discussion about the fact that for some parameters in particular for the female reproductive system, the two negative control groups were not homogenous. Is it possible to estimate to what extend the higher incidence of abnormalities in one of this negative control, usually from the SD exposure arms, might have affected the sensitivity of animals to BPA for the considered exposure scheme?
**Results obtained within BPA treated groups**

In relation with sperm analysis, continuous terminal groups should be analysed. Furthermore, many end points are needed to conclude that BPA alters or not male sperm production: histological and immunohistological analyses of the testis (percentage of apoptotic germ cells, disturbance of the spermatogenic cycle), hormonal assays (testosterone, LH, FSH, PRL, inhibin). Lastly, this study should be completed by functional studies such as quality of ejaculated spermatozoa, libido and fertility of the males. Are the two-year-old males exposed to BPA as fertile as controls?

In relation with histopathology of male reproductive organs showing many BPA-induced disorders, the most striking event is the *pars distalis* hyperplasia. However, it cannot be interpreted as the cellular type involved in this hyperplasia has not been identified. Furthermore, a quantification of this hyperplasia in each animal is required to evaluate its importance.

On a more general level, a quantification of abnormal cells in each animal for each pathology is required: what is the percentage of exfoliated germ cells, lymphocytes, suppurative inflammatory cells?

Although non-neoplastic lesions in mammary gland were significantly increased in female animals continuously exposed to low doses of BPA for 1 year and for 2 years, no modification of the incidence of neoplastic lesions were observed at 2 years in the same conditions; this aspect would need to be discussed in the final report. The low incidence of neoplastic lesions (1/22 ; (4%)%) (see Table 52 p158) under chronic exposure to low doses of BPA (2.5 and 25 μg/bw/day) should be discussed regarding the incidence of spontaneous neoplasms reported in this animal model. In addition, there is a biological significance and a temporal concordance between the increase of atypical foci observed at low dose of BPA CD (2.5 μg/bw/day) after 1 year and the adenocarcinoma observed at the same dosage after 2 years. These findings are in line with what was described by Delclos in the preliminary study in which low number of adenocarcinoma were also described together with non-neoplastic lesions (identified differently than atypical foci). These findings should be examined in details and their biological plausibility considered.

Lastly, the inclusion of the histopathological examination results (non-neoplastic and neoplastic lesions) of the male mammary gland for the control, BPA and EE2 groups would be welcomed.

This study includes as mentioned, TSH, T4, T3 levels measurements, thyroid gland weight and histopathological issues. Whereas these parameters (related to the thyroid gland issue) are not sufficient to conclude on thyroid hormone axis disrupting effects, reproducible effects on follicular cells and Calcitonin producing cells (C-cells) are reported with EE2 or BPA in females and males. For example, EE2 significant dose trends were noted in terminal sacrifice animals for C-cell adenomas or hyperplasia in the thyroid gland respectively for females and males. In the continuous-dose, BPA 2 year females, significant effect on elevated incidence of follicular cell hyperplasia in the 2.5 μg BPA/kg bw/day dose group was shown. Further, in stop-dose BPA one year females, the 2.5 μg BPA/kg bw/day stop-dose group had a higher incidence of C-cell hyperplasia than control.

For males, authors report non-neoplastic lesions in the thyroid glands (interim and terminal sacrifice males for continuous BPA and EE2 and stop-dose BPA). As these histopathological lesions are reported for males, females and for EE2 and BPA groups, the number of histopathological slides used and microscope views illustrating the histopathological lesions would be beneficial for the review.

It should be discussed that due to budget issue, investigations were limited to classical hormonal blood levels and histological issues. The evaluated gland markers cannot predict slight thyroid hormone brain defects and consequent behavior differences. As no cognitive/behavior endpoints
were included in this study, the interpretation of the results on thyroid hormone signaling should consider this limitation. Therefore, this supports the need of finding biomarkers of effects and new sensitive tests (see H2020 call 2018).

As for the thyroid system; few modifications of circulating hormone at 1 year of age and/or histology of the thyroid gland including C cells could be noted from 250 µg/kg/d to higher doses. It is noteworthy however that most of the observations reported in the academic literature regarding an effect of prenatal and early postnatal exposure to BPA on the thyroid are reported for the early postnatal period corresponding to the final maturation step of the thyroid axis and/or on the maternal thyroid status, the two most critical periods in terms of potential adverse effects of the thyroid function relative to brain development. None of those parameters at those stages are currently available in the current study. The presented results indicate some degree of alteration of the thyroid function but should be confirmed at earlier developmental stages.

Statistical data analysis

Statistical analyses of the lesions recorded by histopathology were undertaken with the Poly-K test (K set at 3). The Poly-K test is a method based on the Cochran-Armitage trend test that takes into account the suspected effect to increase statistical power. Following the Poly-K test, two other statistical methods were used to analyse the severity scores of the lesions: (i) a test combining the Jonckheere-Terpstra test (Jonckheere, 1954; Terpstra, 1952) and the Shirley-Williams test (Shirley, 1977; Williams, 1986) and (ii) the RTE test (nonparametric relative treatment; Brunner, Domhof, & Langer, 2002).

Some results might suggest a non-monotonic dose-response relationship. Was the current statistical analysis fitted to analyse such relationship? If not, was any attempt made to analyse properly this type of relationship? Would it be possible to provide individual raw data to run further statistical analyses?

4. CONCLUSIONS AND RECOMMENDATIONS

Some adverse effects were observed on the female reproductive tract at relatively low dose of BPA with endometrial hyperplasia with continuous exposure, vagina hyperplasia… or at higher levels of exposure with ovarian cysts and other ovary abnormalities, accelerated age-related reproductive function failure…. However, some limitations in the method description (physiological and hormonal status at sacrifice, precise methods for histological observations…) and the discussion of the results are required before any conclusion could be made. This remark is also applicable to the other specific endpoints investigated in this core study. Lastly the results of the “academic” part of the project regarding in particular the early postnatal and prepubertal period will be of special interest for a better interpretation of the BPA Clarity studies.

KEYWORDS

Bisphenol A, risk assessment, Draft NTP Research Report on the CLARITY-BPA Core Study
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