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Hepatic Transcript Levels for Genes Coding for Enzymes Associated with Xenobiotic Metabolism are Altered with Age

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ABSTRACT

Metabolism studies are crucial for data interpretation from rodent toxicity and carcinogenicity studies. Metabolism studies are usually conducted in 6 to 8 week old rodents. Long-term studies often continue beyond 100 weeks of age. The potential for age-related changes in transcript levels of genes encoding for enzymes associated with metabolism was evaluated in the liver of male F344/N rats at 32, 58, and 84 weeks of age. Differential expression was found between the young and old rats for genes whose products are involved in both phase I and phase II metabolic pathways. Thirteen cytochrome P450 genes from CYP families 1–3 showed alterations in expression in the older rats. A marked age-related decrease in expression was found for 4 members of the Cyp3a family that are critical for drug metabolism in the rat. Immunohistochemical results confirmed a significant decrease in Cyp3a2 and Cyp2c11 protein levels with age. This indicates that the metabolic capacity of male rats changes throughout a long-term study. Conducting multiple hepatic microarray analyses during the conduct of a long-term study can provide a global view of potential metabolic changes that might occur. Alterations that are considered crucial to the interpretation of long-term study results could then be confirmed by subsequent metabolic studies.

Keywords. Liver; rat; transcriptome; aging; cytochrome P450; xenobiotic metabolism.

INTRODUCTION

Aging has been noted to impact absorption, disposition, metabolism, and excretion of a chemical in both humans (O'Mahony and Woodhouse, 1994; Cusack, 2004) and rodents (Birnbbaum, 1991; Handler and Brian, 1997). However, most toxicology studies examine these processes only in young animals. Because of the central role played by the liver in drug metabolism, we felt it was important to examine the hepatic global gene expression of animals of different ages to assess the potential impact of aging. To accomplish this objective we compared rat hepatic transcripts from livers of untreated male rats collected at 32, 58, and 84 weeks of age using high-density microarrays.

We found age-related alterations in the transcripts of a significant number of genes associated with phase I and phase II reactions of xenobiotic metabolism, which were further validated using quantitative real-time RT-PCR. Importantly, every measured member of the 3A family of cytochrome P450s, which are particularly important for the metabolism of drugs and xenobiotics (Gnerre et al., 2004), had a marked

decrease in transcript levels with age. Immunohistochemistry for Cyp3a2 and Cyp2c11, major hepatic cytochromes in the rat, demonstrated marked decline in the protein levels of these genes with age. Cumulatively, these findings reveal profound age-associated changes in expression of genes whose products are involved in xenobiotic metabolism.

MATERIALS AND METHODS

Animals and Study Design: Male Fischer 344 rats approximately 36 ± 3 days old were supplied by Taconic laboratory animals (Germantown, NY) and were approximately 6 weeks old when placed in study rooms at Battelle Science and Technology International (Columbus, Ohio). The livers were removed from untreated male rats at 6 months (15 total rats), 12 months (10 total rats), and 18 months (13 total rats) for use in this study. The sampling times corresponded to approximately 32, 58, and 84 weeks of age for the rats. This study was approved by Battelle's Institute Animal Care and Use Committee and conducted in accordance with the guide for the care and use of laboratory animals (ILAR, 1996). Details of the animal care and handling for studies conducted at Battelle have been reported previously (Boorman et al., 2005).

The rats were kept 3 per polycarbonate cage until necropsy. The rats were anesthetized with CO₂/O₂, blood samples obtained for serology by cardiac puncture, the abdominal cavity was opened and the portal vein was severed. The left liver lobe was promptly removed and a cross-section was placed in 10% neutral-buffered formalin (NBF). The liver section was embedded, sectioned, and stained with H&E and examined.

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Abbreviations: RT-PCR: reverse transcription polymerase chain reaction; SAM: Statistical analysis of microarray; NTP: National Toxicology Program; CYP: cytochrome P450 family of enzymes.

The remainder of the left lobe was processed for subsequent RNA isolation.

RNA Isolation: The left hepatic lobe was cut into 0.5 cm cubes or smaller and immersed in RNALater (Ambion, Austin, TX) within 4 minutes of necropsy. The tissues were stored in RNALater overnight at $4 \pm 3^\circ\text{C}$, then stored at $-20 \pm 1^\circ\text{C}$ until RNA isolation (within 60 days). Details of the RNA isolation procedures have been previously published (Boorman et al., 2005). Briefly, the RNA samples were frozen at -70°C and shipped to the NTP repository until transfer to Cogenics, a Division of A Clinical Data, for microarray analysis. RNA was isolated from individual rats. Equal amounts of RNA from 63 male sentinel rats (25 rats at 32 weeks of age, 25 rats at 58 weeks of age and 13 rats at 84 weeks of age) were used to form a composite control pool for comparison with the individual rats at each age.

Microarray Hybridizations: One μg of total RNA from either an individual rat or from the pooled sample was amplified and labeled with a fluorescent dye (either Cy3 or Cy5) using the Low RNA Input Linear Amplification Labeling kit (Agilent Technologies, Palo Alto, CA) following the manufacturer's protocol. The amount and quality of the resulting fluorescently labeled cRNA was assessed using a Nanodrop ND-100 spectrophotometer and an Agilent Bioanalyzer. Equal amounts of Cy3- or Cy5-labeled cRNA were hybridized to the Agilent Rat Oligo Microarray (Agilent Technologies, Inc., Palo Alto, CA) for 17 hours, prior to washing and scanning. Data were extracted from the resulting images using Agilent's Feature Extraction Software (Agilent Technologies, Inc., Palo Alto, CA).

Quantitative Real-Time Reverse Transcription Polymerase Chain Reaction (RT-PCR) Analysis of Gene Expression: Real-time reverse transcription polymerase chain reaction (RT-PCR) was performed on hepatic RNA samples from 38 individual rats from the 3 time points. This was done for 10 genes (See Table 1 for probe sequences) that encode for phase I and phase II metabolic enzymes and 1 housekeeping gene (RPL 18). The RT-PCR reactions were performed in duplicate resulting in 836 RT-PCR measurements.

RNA was reverse transcribed into first-strand cDNA using the High-Capacity cDNA Archive kit (Applied Biosystems, Foster City, CA). For each RNA sample, RNA ($2.5 \mu\text{g}$)

in a volume of $50 \mu\text{l}$ was combined with an equal volume of the 2X RT master mix for a total volume of $100 \mu\text{l}$ reaction mixture containing random primers, dNTP mixture, and Multiscribe RT enzyme in a 96-well reaction plate. The plate was incubated for 10 minutes at 25°C and then at 37°C for 2 hours in a 9700 ABI thermocycler. The cDNA was stored at -20°C until further use.

The cDNA was diluted to $2.5 \text{ ng}/\mu\text{l}$ and amplified using MGB Eclipse primer and probe sets (Nanogen Epoch Biosciences, Bothell, WA) on an ABI 7900 Sequence Detection System (Applied Biosystems, Foster City, CA). A 10 ng aliquot of cDNA was loaded into the reaction plate and dried by incubating the plate at 45°C . Master mix was prepared using MGB Eclipse PCR Reagent Kit (Sigma, Saint Louis, MO) and combined with the specified Primer Probe set for a reaction volume of $15 \mu\text{l}$ for each well on a 384 well reaction plate (Table 1: Epoch Biosciences Primer and Probe sequences). Reactions were run in duplicate.

The samples were amplified by incubation for 2 minutes at 50°C , then 2 min at 95°C , followed by 50 cycles of 95°C for 5 seconds, 56°C for 20 seconds and 76°C for 20 seconds. SDS Software version 2.1 (<http://www.dev4pc.com/>) and Microsoft Excel software were used for setup and analysis. Manual threshold values were used and expression of each gene involved in phase 1 or phase 2 metabolism was normalized to RPL18

Immunohistochemistry: Left liver lobes from rats at 32, 58, and 84 weeks of age were examined for Cyp2c11 and Cyp3a2 using immunohistochemical stains. Formalin-fixed paraffin-embedded rat liver tissues were deparaffinized in xylene and hydrated through a graded series of ethanol. The sections were incubated in $3\% \text{ H}_2\text{O}_2$ for 15 minutes to block endogenous peroxidase activity. Antigen retrieval was achieved by using 0.01M citrate buffer, pH 6.0 in an Antigen Decloaker (Biocare Medical, Concord, CA) to unmask antigen sites. The nonspecific staining was blocked for 10 minutes using the Ready-To-Use Serum-Free Protein Block (Dako, Carpinteria, CA). The sections were incubated with rabbit anti-rat Cyp2c11 (Adcam, Cambridge, MA) at a 1:100 dilution for 1 hour at room temperature or rabbit anti-rat Cyp3a2 antibody (Chemicon, Temecula, CA) at a 1:300 dilution for 1 hour. All incubations were conducted at room temperature.

TABLE 1.—Primers for RT-PCR for genes associated with xenobiotic metabolism.

GeneBank #	Symbol	Gene name	Forward primer 5'-3'	Reverse primer 5'-3''
<i>Phase I Enzymes</i>				
NM_012520	Cat	catalase	CCATACTGGATCATGTCTTCCAAA	CTATGTGAAATCA*CTGTGTATCAGC
NM_019184	Cyp2c	cytochrome P450, subfamily 2c	ATTGAGTTGAGTTCCCTGAGTCA	GTA*CTGCATGGGAATGTCCAC
NM_013105	Cyp3a3	cytochrome P450, subfamily 3a, polypeptide 3	CAGATGAAATTAGGGCTTAATCGAC	GTAGTCCCATGAGAATCACCAAAGG
NM_153312	Cyp3a11	cytochrome P450, family 3, subfamily a, polypeptide 11	ACCTTTGCTGATTCTCATGGGA	GGACGAGGACA*TGGTTACTATC
NM_145782	Cyp3a18	cytochrome P450, 3a18	TCAGAAATTCACTGAACAGGCT	GAAGTGTGGAACATA*ACTGGAATCT
<i>Phase II Enzymes</i>				
NM_017013	Gsta2	glutathione S-transferase, alpha type2	CCCAATGTGAAGAAGTTCCTG	AATTGGACAGTGCAGCTCCGCTAA
NM_017014	Gstm1	glutathione S-Transferase mu 1	GATCTCTGCCTACA*TGAAGA	AGCAAGGGCCTACTTGTACTCCAT
NM_177426	Gstm2	glutathione S-Transferase mu 2	CCTACTTAATTGATGGGTCACACA	GGGTGTCCATAGCCTGGTCTCCAA
NM_138974	Gstp2	glutathione S-transferase, pi 2	GATTACAACTTGCTGGACCTG	CACTACTGTTTACCATTGCCGTTGA
NM_031732	Sult1a2	sulfotransferase family 1A, member 2	CAGATACCTTCACTAGCCTGTAAT	GCCTTTGGTTAATGCACAGTGCTCA
<i>Control</i>				
NM_031102 ^a	Rpl18	ribosomal protein L18	GGGTAAGATCCTGACCTTCG	TTGCCAAAGTGTGCGTACACCTCTC

^aDenotes incorporation of modified base to the left.

The bound primary antibody was visualized by incubating tissue sections in the link and label reagents from the Labeled Streptavidin Biotin (LSAB) HRP kit (Dako, Corporation, Carpinteria, CA) for 20 minutes each, followed by a 6-minute incubation in liquid diaminobenzidine (Dako Corporation, Carpinteria, CA) as the color-developing reagent. Slides were counterstained with Harris hematoxylin (Richard Allen Supplies, Kalamazoo, MI), dehydrated through a graded series of ethanols, cleared in xylene and cover slipped with Permount (Fisher Scientific, Springfield, NJ). For negative controls, the primary antibody was replaced with normal rabbit serum (Jackson Immunoresearch, West Grove, PA) diluted to match the protein concentration of the primary antibody.

Analyses of Immunohistochemical Images: A low magnification image (2x lens) of each immunohistochemical slide with either Cyp2c11 or Cyp3a2 antibody was captured using an Olympus DP11 camera. The digital images were imported into ImageJ version 1.37h (<http://rsb.info.nih.gov/ij/>). The brightness and contrast of each image were optimized based on the image's histogram. The k-means clustering plugin (<http://ij-plugins.sourceforge.net/index.html>) for ImageJ was applied to each adjusted image. The parameters entered into the plugin were 4 clusters at 0.1 cluster center tolerance with no randomization seed. To assign a score to each image, the number of pixels in the cluster corresponding to the positive stain was divided by the total number of pixels in the image.

Analyses of Microarray Data: Data from dye reversal hybridizations representing the same individual were combined in the microarray analysis software package Rosetta Resolver version 4.0.0.1.1 (Rosetta Biosoftware, Seattle, WA) using an error-weighted average. Samples taken from the same time points (6, 12, or 18 months) generated consistent measurements overall; however, visualization with PCA allowed identification of an artifact that was due to a difference in a subset of the 6 month samples where the microarrays were conducted at a later time than all other samples (data not shown).

Consistent with previous findings, singular value decomposition corrected for this artifact (Alter et al., 2000; Nielsen et al., 2002). The transformed data did not show the artifact, were more consistent within a time point, and had stronger correlation with the PCR measurements. Therefore, the adjusted data were utilized for all further data analyses. The 2-class unpaired method of Significance Analysis of Microarrays (SAM) was used to identify genes whose expression differed significantly between individual samples collected at 32 versus 84 weeks of age (Tusher et al., 2001). Hierarchical clustering was performed for individuals from 32, 58, and 84 weeks of age using CLUSTER and visualized with TREEVIEW (Eisen et al., 1998).

RESULTS

Microarray Data Analysis to Identify Genes That Vary with Age

The potential for age-related changes in metabolic capacity to impact study results may be underestimated in long-term chemical exposure studies. Because of the central role of the liver in xenobiotic metabolism, we performed gene expression profiling on RNA isolated from individual livers of rats at different ages (32, 58, and 84 weeks) to examine the effect of aging on the expression of hepatic xenobiotic metabolizing enzymes. A 2-class SAM analysis using a 5% false discovery rate was performed on the data from the oldest and youngest animals and identified 2806 differentially expressed transcripts that were either increased (1747) or decreased (1059) at 84 weeks relative to their expression levels at 32 weeks.

An initial review of the list of differentially expressed genes revealed 19 genes encoding enzymes that are members of the cytochrome P450 family. Thirty-six transcripts for key enzymes that catalyze both phase I (Table 2) and phase II (Table 3) reactions are significantly altered in 84-week-old rats compared to 32-week-old animals.

Phase I Enzymes

In this study, there were 21 genes with significantly changed expression levels whose products are involved in

TABLE 2.—Genes for phase I enzymes showing altered expression between 32 and 84 weeks.^a

GeneBank #	Symbol	Gene name	Fold-change
NM_019286	Adh1	alcohol dehydrogenase 1	2.11
NM_134329	Adh7	alcohol dehydrogenase 7	1.65
NM_022273	Aldh9a1	aldehyde dehydrogenase family 9, subfamily A1	1.38
U10698.1	Ces1	carboxyesterase 1	-2.31
NM_133586	Ces2	carboxyesterase 2	1.79
NM_133295	Ces3	carboxyesterase 3	-3.36
D50580.1	Ces5	carboxyesterase 5	1.35
NM_012541	Cyp1a2	cytochrome P450, family 1, subfamily a, polypeptide 2	1.58
NM_012692	Cyp2a1	cytochrome P450, 2a1	3.14
NM_012693	Cyp2a2	cytochrome P450, subfamily 2a, polypeptide 1	-1.64
AF159245.1	Cyp2b13	cytochrome P450, family 2, subfamily b, polypeptide 13, CYP2B21	-1.46
NM_019184	Cyp2c	cytochrome P450, subfamily 2c	-4.59
M13711.1	Cyp2c6	cytochrome P450, subfamily 2c6	2.12
NM_017158	Cyp2c7	cytochrome P450, 2c39	1.97
NM_138515	Cyp2d22	cytochrome P450, family 2, subfamily d, polypeptide 22	1.27
NM_031543	Cyp2e1	cytochrome P450, family 2, subfamily e, polypeptide 1	2.30
NM_013105	Cyp3a3	cytochrome P450, subfamily 3a, polypeptide 3	-3.40
NM_153312	Cyp3a11	cytochrome P450, family 3, subfamily a, polypeptide 11	-34.14
NM_147206	Cyp3a13	cytochrome P450, family 3, subfamily a, polypeptide 13	-3.34
NM_145782	Cyp3a18	cytochrome P450, 3a18	-4.27
NM_012792	Fmo1	flavin-containing monooxygenase 1	-2.10

^aGenes at the 5% False Discovery rate between 84-week and 32-week-old rats using SAM Analysis (Tusher et al., 2001).

phase I reactions (Table 2), which include the hydrolysis, oxidation, or reduction of a compound. Probes for 4 carboxylesterases were present on the microarray. Significantly decreased age-related expression of 2 carboxylesterase family members (Ces1 and Ces3) was observed, while 2 other (Ces2 and Ces5) showed increased transcript levels with age (Table 2). The majority of phase I genes whose transcripts were found significantly altered with age are involved in oxidation-reduction reactions, including alcohol dehydrogenase (Adh), aldehyde dehydrogenase (Aldh), flavin-containing monooxygenase (Fmo), and the cytochrome P450 family (CYP) of enzymes. Adh1, Adh7, and aldehyde dehydrogenase (Aldh9a1) showed increased expression in older rats while flavin-containing monooxygenase (Fmo1) showed decreased expression with age.

The CYP1, CYP2, and CYP3 families of enzymes are generally associated with oxidative metabolism of foreign compounds (Lewis, 2001). Collectively, 13 members of the CYP1, CYP2, and CYP3 families showed altered expression in older rats with 6 genes increased and 7 with decreased transcript levels. The most consistent change was in the Cyp3a family where all 4 members showed decreased expression with age (Table 2). Furthermore, hierarchical clustering of the phase I enzymes demonstrating age-related changes reveals the CYP3A family of genes, Ces1, Ces3, and Fmo1 grouping together and characterized by decreased expression in the 84-week-old rats (Figure 1).

Phase II Enzymes

Elimination of some compounds can occur based solely on the action of phase I enzymes; however, others need further biotransformation by phase II enzymes. Often an oxygen molecule is added by a phase I oxidation creating a polar metabolite. Phase II reactions typically involve the conjugation of the polar function group creating a water-soluble compound that is excreted. Thirteen genes whose products are involved in phase II reactions showed significant alterations in transcript levels at 84 weeks of age (Table 3). The biotransformation processes included glucuronidation (Ugt1a6, Ugt2b5), sulfation (Sult1a2, Sult1c2), acetylation (Nat8), and glutathione conjugation (Gsta2, Gsta5, Gsr, Gss, Gstt1, Gstp2).

The expression of individual members of the same family did not all change uniformly with age (Figure 1). For example transcripts for Ugt1a6 increased with age while transcripts for Ugt2b5 decreased with age. Genes whose products

are associated with glutathione conjugation including Gsta5, Gss, and Gstt1 had lower transcript levels in young rats while Gsta2 transcripts were increased in the same animals. There were nearly equal numbers of increased (7) and decreased (6) transcript levels for genes whose products are associated with phase II metabolic reactions.

RT-PCR Analysis of Selected Transcripts

We used real-time RT-PCR to analyze transcripts for 5 phase I enzymes (Figure 2) and for 5 phase II enzymes (Figure 3) using the same RNA samples from the microarray analysis. There was little variability in the RT-PCR replicate analysis and excellent correlation ($r = 0.86$) between RT-PCR and microarray results from the individual animals. The RT-PCR analysis included glutathione S-transferase, mu type 2 (Gstm2) that was not represented on the microarray. All phase I and phase II transcripts levels that were measured by RT-PCR were significantly ($p < 0.01$) decreased at 84 weeks compared to 32-week-old rats.

Histochemical Analysis of Selected Cytochrome P450s

Liver sections from 32, 58 and 84 week-old rats were analyzed for Cyp2c11 and Cyp3a2 protein using primary antibodies with streptavidin biotin for visualization. Images were captured from each section and the intensity of immunohistochemical staining was analyzed using a computer visualization program (Table 4). Cyp2c11 and Cyp3a2 protein was found in the cytoplasm of hepatocytes around the terminal hepatic venule (THV) and in the hepatocytes immediately adjacent to the large hepatic veins. Stain was absent from biliary epithelial cells, nonparenchymal cells and the occasional inflammatory foci for Cyp2c11 but moderate staining of biliary ductal cells was seen for Cyp3a2. The centrilobular staining decreased markedly at 84 weeks (Figure 4) but focal areas of vacuolated hepatocytes that may have represented foci of alteration stained intensely for Cyp3a2, but did not stain for Cyp2c11.

DISCUSSION

Aging has been noted to impact absorption, disposition, metabolism, and excretion of a chemical in both humans (O'Mahony and Woodhouse, 1994; Cusack, 2004) and rodents. Although it has been previously shown that aging decreases hepatic cytochrome p450 protein content in the rat (Handler and Brian, 1997), this is the first study to provide a global assessment of age-related changes in genes that encode

TABLE 3.—Genes for phase II enzymes showing altered expression between 32 and 84 weeks.^a

GeneBank #	Symbol	Gene name	Fold change
NM_012844	Ephx1	epoxide hydrolase 1	2.38
NM_017013	Gsta2	glutathione S-transferase, alpha type2	-2.17
NM_031509	Gsta5	glutathione S-transferase, a5	1.77
NM_053906	Gsr	glutathione reductase	1.57
NM_012962	Gss	glutathione synthetase	1.59
NM_053293	Gstt1	glutathione S-transferase theta 2	-1.54
NM_138974	Gstp2	glutathione S-transferase, pi 2	2.35
NM_022635	Nat8	N-acetyltransferase 8 (camello like)	-3.17
NM_017000	Nqo1	NAD(P)H dehydrogenase, quinone 1	1.87
NM_031732	Sult1a2	sulfotransferase family 1a, member 2	-2.12
NM_133547	Sult1c2	sulfotransferase family cytosolic, 1c, member 2	-1.43
NM_130407	Ugt1a6	UDP glycosyltransferase 1 family, polypeptide a6	1.48
M33747.1	Ugt2b5	UDP glycosyltransferase 2 family, member 5	-1.74

^a Genes at the 5% False Discovery rate between 84-week and 32-week-old rats using SAM Analysis (Tusher *et al.*, 2001).

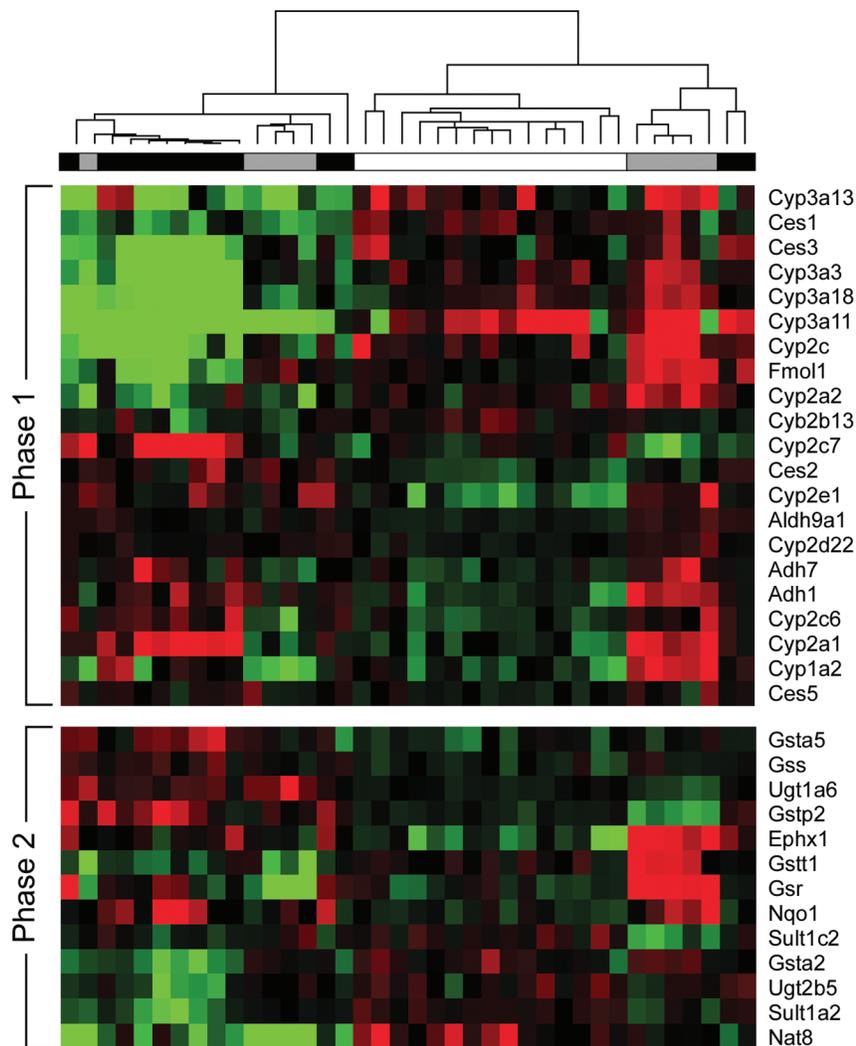


FIGURE 1.—Heat maps of 38 individual rats at 32 weeks (white bar), 58 weeks (gray bar) and 84 weeks (black bar) of age hybridized to a pool of 63 rats from the 3 time points. Clustering is shown for the differentially expressed Phase I and Phase II enzymes shown in Tables 2 and 3. Decreased expression is shown in green while increased expression is shown in red. Gene symbols for selected known genes are to the right of the insets.

enzymes involved in both phase I and phase II metabolic reactions and suggests that the decreased protein content reported by others in the rat (Handler and Brian, 1997) is transcriptionally regulated. Furthermore, this study raises the question of whether data on biotransformation pathways obtained from young animals is completely relevant when analyzing data from long-term studies.

In evaluating this data, some caution is necessary since protein content is not always reflective of transcript levels (Wauthier et al., 2004b). Further, factors unrelated to enzyme content such as absorption, blood flow, concentrations of substrates, etc. also may have a significant impact upon how drug metabolism is affected by aging (Handler and Brian, 1997). In spite of these limitations, the microarray results presented here and the confirmatory real-time RT-PCR and immunohistochemistry results clearly demonstrate that the aging process has a significant impact on hepatic transcript and protein levels for many genes associated with drug metabolism.

Phase I metabolism reactions involve the hydrolysis, reduction, and oxidation of xenobiotics (Parkinson,

2001). In this study we found altered transcript levels for 21 genes whose products are associated with phase I metabolism including dehydrogenases, carboxylesterases, a flavin-containing monooxygenase, and numerous genes that encode enzymes of the cytochrome P450 family.

Alcohol and aldehyde dehydrogenases play an important role in the metabolism of both endogenous and exogenous alcohols and aldehydes (Cheung et al., 2003). We found increased expression for alcohol dehydrogenase1 (Adh1), Adh7 and aldehyde dehydrogenase 9a1 (Aldh9a1) in older rats. In male rats metabolism of 2-butoxyethanol (BE) starts to increase by 12 months of exposure and is up 2-fold by 18 months (Lee et al., 1998). Adh1 preferentially metabolizes short-chain alcohols such as BE (Lockley et al., 2005). It is interesting that Adh1 transcript levels in the current study start to increase at 12 months and are up 2-fold by 18 months.

Adh genes also contain sterol regulatory elements (SRE) in their promoters (He et al., 2006). Adh and Aldh are involved in the pathway leading to fatty acid biosynthesis. We have observed that other genes associated with fatty acid and lipid

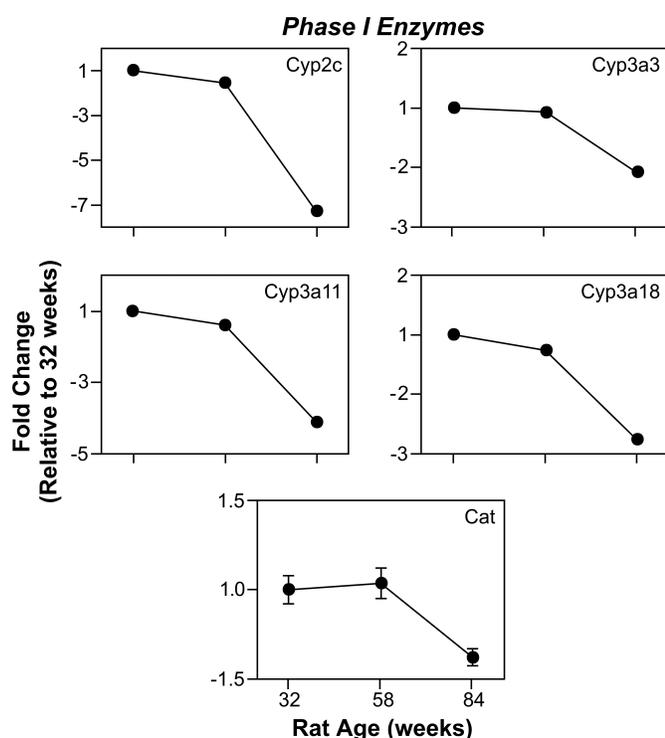


FIGURE 2.—Graphic presentation of RT-PCR reactions for 5 genes whose products are associated Phase I enzymes. The solid lines are mean values (\pm SEM) with 15 rats at 32 weeks, 10 at 58 weeks and 13 at 84 weeks. The values were normalized to the housekeeping gene Rpl 18 and then expressed relative to the mean values of the rats that were 32 weeks of age. Each assay was run in duplicate. Where no \pm bars appear, the values were smaller than the symbol for the mean.

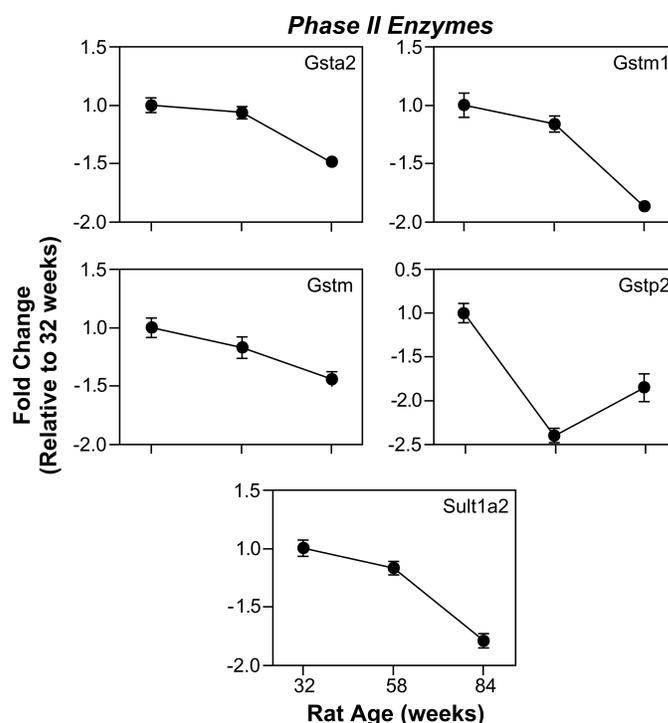


FIGURE 3.—Graphic presentation of RT-PCR reactions for 5 genes whose products are associated Phase II enzymes. The solid lines are mean values (\pm SEM) with 15 rats at 32 weeks, 10 at 58 weeks, and 13 at 84 weeks. Each assay was run in duplicate. The values were normalized to the housekeeping gene Rpl 18 and then expressed relative to the mean values of the rats that were 32 weeks of age. Each assay was run in duplicate. Where no \pm bars appear, the values were smaller than the symbol for the mean.

metabolism demonstrate an age-related increase in expression levels (Mori et al., unpublished data). This may explain the increased expression for these dehydrogenases since they appear, in part, to be under the same sterol regulatory element control.

Carboxylesterases are well-characterized drug hydrolyzing enzymes (Landowski et al., 2006) that are important in detoxification of organophosphorus pesticides such as parathion (Karanth and Pope, 2000). In this study carboxylesterase 1 (Ces1) and Ces3 showed a greater than 2-fold decrease in hepatic transcript levels at 84 weeks of age while Ces2 and Ces5 showed increased mRNA levels with age (Table 2). The variation in the different Ces isoforms with age may explain why the total hepatic carboxylesterase activity

is similar between adult and aged rats (Karanth and Pope, 2000). Aged rats are markedly more sensitive to parathion perhaps related to the lower carboxylesterase plasma levels in aged rats (Karanth and Pope, 2000). The toxicological importance of the carboxylesterases in catalyzing the hydrolysis of a variety of drugs (Satoh, 2005) including their role in the detoxification of widely used pyrethroid insecticides (Huang et al., 2005) and organophosphorus pesticides (Karanth and Pope, 2000) highlights the potential importance of understanding these age-related transcript changes.

The flavin-containing monooxygenases (FMO) are considered to have evolved to protect mammals from lipophilic and nucleophilic plant materials (Cashman, 2005). FMO-catalyzed oxidation converts lipophilic compounds to

TABLE 4.—Image analysis for Cyp3a2 and Cyp2c11 positive staining protein at 32, 58, and 84 weeks.^a

Animal	Cyp3a2 (3a11)			Cyp2c11		
	32 Weeks	58 Weeks	84 Weeks	32 Weeks	58 Weeks	84 Weeks
1 ^a	12.9 ^b	10.3	9.7	33.0	19.3	12.7
2	13.1	10.1	0.4	18.2	46.2	4.6
3	7.3	24.7	5.5	20.4	44.4	6.5
4	8.9	28.2	6.5	17.7	18.5	40.9
5	—	25.5	2.7	—	34.0	3.4
Mean (\pm SD)	10.6 \pm 2.9	19.8 \pm 8.8	5.0 \pm 3.6	22.3 \pm 14.5	32.5 \pm 13.2	13.6 \pm 15.7

^aThe animal number corresponds to pairing for the two stains at the same time point, but not the same animal across time.

^bPercent positive stain was obtained by the number of pixels in the cluster corresponding to the positive stain divided by the total number of pixels in the image. 100 \times .

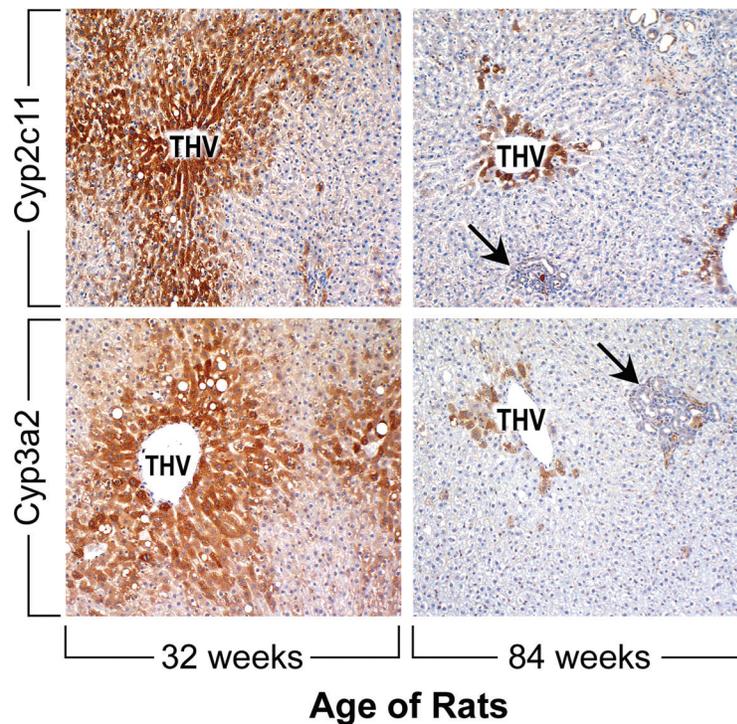


FIGURE 4.—Immunohistochemical reactions for Cyp2c11 and Cyp3a2 (Cyp3a11) protein in the livers of male rats at 32 and 84 weeks of age. Rabbit anti-rat Cyp2c11 was used to visualize the Cyp2c11 protein that appears as dark brown granules in the cytoplasm of hepatocytes about the terminal hepatic venule (THV) in 32-week-old rats. The bound antibody was visualized using a Streptavidin Biotin kit with diaminobenzidine as the color developing reagent. At 84 weeks only a few hepatocytes around the THV contain intense brown cytoplasmic granules. Rabbit anti-rat Cyp3a2 was used to visualize the Cyp3a2 protein that appears as light brown granules in the cytoplasm of hepatocytes about the terminal hepatic venule (THV) in 32-week-old rats. In the 84-week-old rats only occasional hepatocytes around the THV contain brown cytoplasmic granules. The slides were counter stained with Harris hematoxylin. In the 84-week-old rats, occasional foci of proliferating biliary epithelium (arrow) are seen.

hydrophilic metabolites facilitating excretion and generally reducing the pharmacological activity of the compound (Cashman, 2005). We found a greater than 2-fold decline in Fmo1 transcript levels between the young and old F344 rats. Fmo1 levels have also been reported to decline with age in male Wistar rats (Okamoto et al., 1994; Sanz et al., 2002). The decrease in the hepatic FMO activity may explain the decreased nicotine metabolism with age in rats (Okamoto et al., 1994).

The majority of genes for which the transcript levels declined with age encode enzymes that are members of the cytochrome P450 family (Table 2). We focused on the CYP1, CYP2, and CYP3 families since they are the major P450 families involved in phase I xenobiotic metabolism (Hoffman et al., 2001). The Cyp1 family plays an important role in activation of exogenous procarcinogens (Nebert and McKinnon, 1994) and is composed of at least 2 subfamilies, Cyp1a and Cyp1b (Kawajiri and Hayashi, 1996).

Three members of the CYP1 family were represented on the microarray including Cyp1a1, Cyp1a2, and Cyp1b1, but only Cyp1a2 showed increased transcript levels with age. These findings are consistent with the finding that increased hepatic Cyp1a2, but not Cyp1a1, transcript levels were found in aged male mice (Mikhailova et al., 2005). Our findings and those of Mikhailova et al. (2005) suggest that metabolic studies in young rodents may underestimate the toxic exposure in rats and mice in the later stages of a 2-year study for polycyclic aromatic hydrocarbons and heterocyclic amines

that are converted to highly reactive intermediates by Cyp1a2 (Nebert and McKinnon, 1994).

The large Cyp2 family contains multiple subfamilies involved in the metabolism of endogenous steroid and xenobiotics (Hoffman et al., 2001). Cyp2a2, Cyp2c11, and Cyp3a11 (3a2) account for 60–70% of the total P450-expressed in the male rat liver (Dhir and Shapiro, 2003). Six of the 19 members of the CYP2 family 3 in the microarray analysis showed increased expression in the older rats while 3 had an age-related decrease in expression. Interestingly, there were age-related effects on gender-specific CYP2 family members.

The rat male specific isoforms Cyp2a2 (Chang and Waxman, 1996) showed decreased expression with age whereas Cyp2a1, and Cyp2c7 considered to be female predominant isoforms (Chang and Waxman, 1996; Ohhira et al., 2006) demonstrated increased expression. The functional feminization of drug-metabolizing activities in the liver of the aged male rat liver (Fujita et al., 1990) is thus reflected in the observed transcript changes. Cyp2c11 a major male specific hepatic P450 enzyme that may explain significant sex differences in the metabolism of tributyltin (Ohhira et al., 2006), was not included on our microarray. Therefore immunohistochemical methods were used to analyze for Cyp2c11 protein levels. The dramatic decline in staining for Cyp2c11 protein levels is consistent with the decline in Cyp2c11 mRNA and Cyp2c11 activity found with age in male Sprague-Dawley rats (Agrawal and Shapiro, 2003; Dhir and Shapiro, 2003).

Cyp2c6 also showed increased transcript levels with age and is considered to be a female-predominant isoform by some (Agrawal and Shapiro, 2003) while others find no gender differences (Imaoka et al., 1991). One possible explanation is that Imaoka's studies involved Wistar rats while Agrawal's studies used Sprague–Dawley rats. Hepatic Cyp2c6 constitutive and phenobarbital-induced expression is highly strain specific (Larsen et al., 1994). The differences in isoform-specific metabolism closely parallel specific P450 mRNA levels suggesting that transcript levels may predict metabolism differences (Larsen et al., 1994).

Many toxicologically important compounds including ethanol, carbon tetrachloride, and acetaminophen are biotransformed to their toxic metabolite by Cyp2e1 enzyme (Bai and Cederbaum, 2006). We found an approximate 2-fold increase in hepatic Cyp2e1 transcript levels with age in rats. CYP2E1 activity as measured by chlorzoxazone metabolism shows an age-associated increase in humans (Bebia et al., 2004) but a decrease in rats between 8 and 18 months of age (Wauthier et al., 2004b). In the same study in male rats both Cyp2e1 mRNA and CYP2E1 hepatic protein content remained fairly constant (Wauthier et al., 2004b).

The authors suggest that age-related posttranslational modifications might diminish the catalytic efficiency of the enzyme (Wauthier et al., 2004b). Studies using rat liver microsomes from Fischer 344 rats between 3 and 26 months of age also show CYP2E1 activity declining with age (Warrington et al., 2004a). This study was based on chlorzoxazone biotransformation that may be mediated by both CYP2E1 and CYP1A1 (Warrington et al., 2004a). As noted previously, we observed no significant changes in Cyp1a1 transcript levels.

The CYP3A family is the most abundant of the hepatic cytochrome P450 enzymes in both rats and humans (Kawai et al., 2000; Gnerre et al., 2004). The 3A enzymes are major catalysts in the oxidative biotransformation of steroids, drugs and xenobiotics (Kawai et al., 2000). Cyp3a3, Cyp3a11, Cyp3a13, and Cyp3a18 are the official symbols for the 4 members of the Cyp3a subfamily in the rat (Entrez Gene at <http://www.ncbi.nlm.gov/entrez/>). Unfortunately the terminology is not consistent in the rat, Cyp3a1 and Cyp3a23 are common aliases for Cyp3a3, Cyp3a2 is an alias for Cyp3a11, and Cyp3a9 for Cyp3a13, but Cyp3a18 generally appears as Cyp3a18. In our study the transcript levels for all 4 measured family members declined by 3- to 30-fold between 32 and 84 weeks of age. The greater than 3-fold decrease in Cyp3a3 expression with age by microarray analysis was confirmed by RT-PCR. This is in contrast to a study in male Sprague–Dawley rats where no age-related change in hepatic expression for Cyp3a3 (Cyp3a1) was found (Agrawal and Shapiro, 2003).

Similar to our findings 2 other studies using RT-PCR noted a decrease in Cyp3a3 (Wauthier et al., 2004a; Peng et al., 2005) with age in male Wistar rats. It is unclear whether Cyp3a3 behaves differently in Sprague–Dawley rats or whether the radioactive probe technology lacked the sensitivity to detect an age-related decline in Cyp3a3 (Agrawal and Shapiro, 2003). The transcript level for Cyp3a11, the rodent ortholog of the most abundant cytochrome P450 (Cyp3a4) in the human liver (Zollner et al., 2006), showed a 34-fold decrease between 32 and 84 weeks. This was supported by immunohistochemical finding of abundant protein in cen-

trilobular hepatocytes of young rats with almost no Cyp3a11 in older rats. Cyp3a13 transcript was reduced 3-fold in 84-week-old rats.

While it has been shown that Cyp3a13 (also known as Cyp3a9) increases with age in male rats (Kawai et al., 2000), this is the first demonstration, to our knowledge, that Cyp3a13 and Cyp3a18 expression levels decline with age in male rats. Decreased hepatic enzyme activity for the CYP3A family was seen in older male Fischer-344 rats compared to 3–4-month-old animals as well as transcript levels for Cyp3a3 and Cyp3a11 (Warrington et al., 2004b). Our results confirm decreased transcript levels for these 2 members of the Cyp3A family and extend the findings to the other 2 members of this family. Our results and those of Warrington et al. (2004b) suggest that the male rat may have a declining ability to metabolize some xenobiotics during the second year of a study.

Phase II reactions also play an important role in increasing the xenobiotic's hydrophilicity and promoting the excretion of the chemical through conjugation reactions, typically to glucuronic acid, sulfates, or glutathione. In rodents, glucuronidation catalyzed by glucuronosyltransferases is a major pathway of xenobiotic metabolism (Tephly, 1990). Two members of this class (Ugt1a6 and Ugt2b5) showed changes in expression with aging (Table 3). Changes in phase II reactions involving glucuronyltransferase activity have also been reported in aging male Sprague–Dawley rats (Handler and Brian, 1997). Age-related decreases in the ability of rat liver microsomes to glucuronidate ketoprofen have been reported (Satterwhite and Boudinot, 1992).

Sulfotransferases generally detoxify xenobiotics by forming excretory water-soluble sulfates but also have the potential to form carcinogenic metabolites from natural plant components such as safrole (Miller, 1994; Klaassen et al., 1998). We found decreased transcript levels for Sult1a2 and Sult1c2 in old rats. Aging has been shown to decrease sulfotransferase activity toward acetaminophen in 25 month-old Fischer rats as compared to 5- or 14-month-old animals (Galinsky et al., 1986). Sult1a2 has been shown to be important in the activation of suspected human carcinogens (Arlt et al., 2005).

We found 6 genes involving glutathione conjugation whose transcripts changed with age. Glutathione S-transferase activity has been reported to decline or remain the same in rats between 6 and 30 months of age, depending on the substrate used to measure activity (van Bezooijen, 1984; Chen et al., 1994). Others report a slight increase with age for glutathione S-transferase activity in the liver of male Wistar rats (Sanz et al., 2002). It is interesting to note that there is a marked decline in the ability of a xenobiotic to induce glutathione-S-transferase activity in 18-month-old rats compared to 6- and 12-month-old animals (Sanz et al., 2002). Our results and those of others (Chen et al., 1994; Sanz et al., 2002) suggest phase II metabolism involving glutathione conjugation may change with age for certain xenobiotics.

In conclusion, we have found age-associated changes in the hepatic transcriptome in untreated rats for many genes whose products are associated with xenobiotic metabolism including Cyp3a13 and Cyp3a18 for which age-related declines have not been previously reported. These findings are consistent with other studies that show age-related changes in metabolic capacity. The present study suggests that many age-related metabolic changes are transcriptionally regulated.

mRNA transcript levels are useful in the quantification of specific cytochrome P450 isoforms (Caron et al., 2005) and mRNA levels are generally in agreement with enzyme activities for the isoforms (Agrawal and Shapiro, 2003).

Transcript levels for the 58-week-old rats had features of both the 32-week-old and 84-week-old rats. Housing was identified as a variable for the 58-week-old rats and could be a contributing factor to this finding. This variable is currently under study. The RT-PCR and immunohistochemistry results suggest that most of the metabolic changes occur after 12 months of age. We suggest that including a hepatic microarray assessment in both treated and untreated animals during the second year of a study would provide global metabolic information of possible effects of both aging and xenobiotic induction. The potential metabolic alterations suggested by microarray results could then be verified for a more informed interpretation of the toxicity and cancer that may appear only in the second year of a long-term study.

SUPPLEMENTARY MATERIAL

The microarray data from the age comparisons is available at GEO at (<http://www.ncbi.nlm.nih.gov/geo/>) accession number GSE4270).

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