Effect of Aging on mRNA Profiles of Drug Metabolizing Enzymes and Transporters in Livers of Male and Female Mice

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Abbreviations
Abc, ATP-binding cassette; Adh, alcohol dehydrogenase; AhR, aryl hydrocarbon receptor; Aldh, aldehyde dehydrogenase; Bcrp, breast cancer resistant protein; CAR, constitutive androstane receptor; Ces, carboxylesterase; Comt, catechol-O-methyl transferase; Cyp, cytochrome P450; Ent, equilibrative nucleoside transporter; Fmo, flavin-containing monooxygenase; Gapdh, glyceraldehyde 3-phosphate dehydrogenase; Gst, glutathione S-transferase; HNF, hepatocyte nuclear factor; Mate, multidrug and toxin extrusion; Mdr, multidrug resistance protein; Mgst, microsomal glutathione S-transferase; Mrp, multidrug resistance-associated protein; Nat, N-acetyltransferase; Nqo1, NAD(P)H: quinone oxidoreductase; Oat, organic anion transporter; Oatp, organic anion-transporting polypeptide; Oct, organic cation transporter; Papss, 3'-phosphoadenosine 5'-phosphosulfate synthetase; Pon, paraoxonase; Por, cytochrome P450 reductase; PPAR, peroxisome proliferator-activated receptor; PXR, pregnane X receptor; RXR, retinoid X receptor; Sult, sulfotransferase; Ugp, UDP-glucose pyrophosphorylase; Ugt, UDP-glucuronosyltransferase; XPG, xenobiotic processing gene
ABSTRACT

Aging is a physiological process characterized with progressive functional decline in various organs over time. In order to reveal possible molecular mechanisms of altered xenobiotic disposition and toxicity in the elderly, age-dependent mRNA profiles of 101 xenobiotic processing genes (XPGs) were characterized in livers of male and female mice from 3 to 27 months of age, including 7 uptake transporters, 41 phase-I enzymes, 36 phase-II enzymes, 10 efflux transporters, and 7 transcription factors. Gender differences across the lifespan (significant at five ages or more) were observed for 52 XPGs, including 15 male-predominant (e.g. Oatp1a1, Cyp3a11, Ugt1a6a, Comt, and Bcrp) and 37 female-predominant genes (e.g. Oatp1a4, Cyp2b10, Sult1a1, Ugt1a1, and Mrp3). During aging, the mRNAs of 44% of the 101 XPGs changed in male mice and 63% changed in female mice. In male mice, mRNAs of 40 XPGs (e.g. Oatp1a1, Ces2c, Gstm4, Gstp1, and Ces1e) were lower in aged mice (over 21 months of age), whereas mRNAs of 4 XPGs (e.g. Oat2 and Gstm2) were higher in aged mice. In female mice, mRNAs of 43 XPGs (e.g. Oatp1a1, Cyp1a2, Ces1f, Sult3a1, Gstt2, Comt, Ent1, Fmo3, and Mrp6) were lower in aged mice, whereas mRNAs of 21 XPGs (e.g. Oatp1a4, Nqo1, Adh7, Sult2a1/2, Gsta1, and Mrp4) were higher in aged mice. In conclusion, 51% of the 101 XPGs have gender differences in liver mRNAs across the lifespan of mice, and the mRNAs of 40% of the XPGs are lower in aged male mice and 43% are lower in aged female mice.
INTRODUCTION

Aging is characterized with declining physiological functions of various organs and increased incidence of multiple concomitant diseases such as diabetes, hypertension, and arthritis (Sandhiya and Adithan, 2008). The older population, described as people over the age of 65, made up 12.8% of the U.S. population during the past decade. This population is the recipient of approximately 33% of all prescription drugs and 40% of all nonprescription drugs. The elderly have been reported to have changes in the absorption, distribution, metabolism, and excretion (ADME) of many drugs, such as non-steroid anti-inflammatory drugs, antihypertensive, anticonvulsant, and psychiatric drugs (Cusack, 2004). In rodent studies, increased susceptibility to environmental chemicals has been observed during aging (Birnbaum, 1991).

Liver is the major organ for the detoxification and elimination of xenobiotics, such as drugs and environmental chemicals. Diverse xenobiotic processing genes (XPGs) are expressed at high levels in liver, including uptake transporters, phase−I enzymes, phase−II enzymes, and efflux transporters. Uptake transporters remove xenobiotics from the portal blood and transfer them into liver for metabolism. Organic anion-transporting polypeptides (Oatps/Slc22a) and organic anion transporter 2 (Oat2/Slc22a7) are important transporters for organic anions, whereas organic cation transporter 1 (Oct1/Slc22a1) is important for organic cations. Equilibrative nucleoside transporter 1 (Ent1/Slc29a1) mediates the uptake of nucleosides. Xenobiotics can be oxidized, reduced, or hydrolyzed by various phase−I enzymes, such as cytochrome P450s (Cyps), cytochrome P450 reductase (Por), NAD(P)H:quinone oxidoreductase 1 (Nqo1), flavin-containing monooxygenases (Fmos), alcohol dehydrogenases (Adhs), aldehyde
dehydrogenases (Aldhs), carboxylesterases (Cess), and paraoxonases (Pons). The phase-I metabolites usually are not hydrophilic enough to be excreted. Therefore, they are conjugated to increase hydrophobicity. Phase-II enzymes, namely the sulfotransferases (Sults), UDP-glucuronosyltransferases (Ugts), glutathione S-transferases (Gsts), catechol-O-methyl transferase (Comt), and N-acetyltransferases (Nats), catalyze conjugating reactions with sulfate, glucuronic acid, glutathione, methyl, or acetyl groups, respectively. Xenobiotics and/or metabolites are to be excreted either into bile by efflux transporters, such as the multidrug resistance-associated protein 2 (Mrp2/Abcc2), breast cancer resistant protein (Bcrp/Abcg2), multidrug and toxin extrusion 1 (Mate1/Slc47a1), multidrug resistance protein 2 (Mdr2/Abcb4), and transporter heterodimer Abcg5/g8, or into blood by the multidrug resistance-associated proteins (Mrp/Abcc 3, 4, and 6) and transporter Abca1 (Klaassen and Lu, 2008). The changes of XPG expression during aging may help explain differences in drug pharmacokinetics and toxicity in the elderly.

Many of the XPGs are regulated by transcription factors, such as the aryl hydrocarbon receptor (AhR), constitutive androstane receptor (CAR/Nr1I3), pregnane X receptor (PXR/Nr1I2), peroxisome proliferator-activated receptor alpha (PPARα/Nr1c1), retinoid X receptor alpha (RXRα/Nr2b1), and hepatocyte nuclear factors (HNF1α and HNF4α) (Klaassen and Aleksunes, 2010). Many of these transcription factors can be activated by xenobiotics to regulate the transcription of drug metabolizing enzymes and transporters (Klaassen and Slitt, 2005).

Gender differences in the baseline expression of many XPGs are regulated by sex hormones or sex-dependent growth hormone patterns (Waxman and O'Connor, 2006).
Gender-divergent expression regulated by growth hormone secretion was reported for Cyps (Pampori and Shapiro, 1999), Sults (Liu and Klaassen, 1996; Alnouti and Klaassen, 2006), Ugts (Buckley and Klaassen, 2009), and Gsts (Srivastava and Waxman, 1993; Knight et al., 2007), as well as xenobiotic transporters (Tanaka et al., 2005; Cheng et al., 2006; Maher et al., 2006). Because of the altered sex hormones and growth hormone levels in old age (Rudman et al., 1990; Bjornerem et al., 2004), it is important to investigate the gender-differences of XPG expression during aging.

Previous studies concerning age-dependent expression of xenobiotic metabolism genes have been restricted to only a couple of age groups (Handler and Brian, 1997; Wauthier et al., 2004; Mori et al., 2007; Lee et al., 2008), and thus possible changes of XPG expression could have been missed. Previous reports were primarily in rats, with limited data available for mice. Mice are a common laboratory model due to the availability of well-characterized mouse genome and genetically engineered mice. In addition, previous studies seldom report gender differences of XPG mRNAs during aging. Taken together, the present study was designed to investigate the comprehensive age-dependent mRNA profiles of XPGs in livers of both male and female C57BL/6 mice with aging.
MATERIALS AND METHODS

Animals. Male (M) and female (F) C57BL/6 mice of various ages were purchased from the National Institute of Aging (Bethesda, MD) and acclimated for at least one month before tissue collections. Mice were housed in an Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC)-accredited facility with a 14-h light/10-h dark-cycle, temperature-, and humidity-controlled environment and given ad libitum access to water and standard rodent chow (Harlan Teklad 8604; Harlan Teklad, Madison, WI). At 3, 6, 9, 12, 15, 18, 21, 24, and 27 months of age, mice (n=5-7) were anesthetized with pentobarbital (50 mg/kg, i.p.). About 10-min later, when the mouse was well anesthetized, the liver was removed, snap-frozen in liquid nitrogen, and stored at -80°C. To decrease the mRNA variation of drug processing genes by circadian rhythm (Zhang et al., 2009), livers were collected between 9:00 and 12:00 in the morning. These studies were approved by the Institutional Animal Care and Use Committee at the University of Kansas Medical Center.

Total RNA Isolation. Total RNA was isolated from liver tissue using RNA Bee reagent (Tel-Test Inc., Friendswood, TX) following the manufacturer's protocol. The concentration of total RNA in each sample was quantified spectrophotometrically at 260 nm.

Multiplex Suspension Assay. The mRNA expression of genes of interest in liver was determined by Panomics 2.0 QuantiGene Plex technology (Panomics/Affymetrix Inc., Fremont, CA), following the manufacturer's protocol. Individual gene information can be found on Panomics Web site (http://www.panomics.com) with Panel numbers 21095, 21152, 21153, 21174 and 21175. The mRNA of target genes were normalized to
housekeeping gene Gapdh.

**Branched DNA Assay.** The branched DNA (bDNA) assay (Quantigene High Volume bDNA Signal Amplification Kit; Panomics/ Affymetrix Inc., Fremont, CA) was used to quantify the mRNA of Comt (because this gene was not included in any pre-designed panels of the multiplex suspension assay). The assay was performed as described previously (Cheng et al., 2005). Probe sets (containing capture extenders, label extenders, and blockers) specific to Comt were designed using ProbeDesigner software (Bayer Corp., Emeryville, CA), shown in Supplementary Table 1.

**Real-time Quantitative Reverse Transcription PCR (real-time qRT-PCR) Analysis.** Total RNA was transcribed to single-stranded cDNA using High Capacity cDNA Reverse Transcription Kit 1001073 (Applied Biosystems, Foster City, CA). Reverse transcription products were then amplified by PCR, using Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) and PCR primers (Integrated DNA Technologies, Coralville, IA). The mRNAs of GAPDH in livers of male and female mice at 3 and 27 months of age were quantified. The primer sequences for GAPDH (NM_008084.2) were 5'-aactttgcattgtggaagg-3' (forward) and 5'-ggatgcagggatgtttct-3' (reverse).

**Statistical Analysis and Hierarchical Clustering.** Data are presented as mean ± SEM. Asterisks (*) represent gender differences between male and female mice, determined by Student's t-test (p<0.05). Daggers (†) and double daggers (‡) represent age differences (p<0.05) compared to 3 months of age in male and female mice, respectively, determined by one-way ANOVA, followed by Duncan's post-hoc test. Hierarchical clustering of XPGs that had mRNA changes with aging (p<0.05, ANOVA)
was performed using JMP 8.0 software (SAS Institute, Cary, NC). High mRNA abundance was represented in red color, whereas low mRNA abundance was in blue. The mRNAs were normalized within each gene, so the relative color intensity is not comparable between genes.
RESULTS

In the present study, data at 3 and 27 months of age were compared most often, because they represent young adult and senescent ages respectively. The mRNAs of XPGs were normalized to Gapdh, which is the most commonly used house-keeping gene. The mRNA of Gapdh was quantified by real-time qRT-PCR. As shown in Supplementary Fig. 1, Gapdh mRNA remained relatively constant during aging and did not have gender differences.

**Uptake Transporters during Aging.** Among the uptake transporters that had decreased mRNAs with age, Oatp1a1 mRNA decreased most markedly between 3 and 27 months of age (M: 55%; F: 100%), and Oct1 mRNA decreased slightly between 3 and 27 months (M: 28%; F: 24%) (Fig. 1). In contrast, Oatp1a4 mRNA increased 96% between 3 and 27 months in male mice, and 30% between 3 and 18 months in female mice. Oatp2b1 mRNA at 9-27 months was higher than 3 months in female mice, whereas in male mice the changes were not obvious. Ent1 mRNA increased from 3 to 9 months and tended to decrease thereafter in female mice, whereas in male mice it remained constant. The mRNAs of Oatp1b2 and Oat2 remained relatively constant with aging. The mRNAs of most uptake transporters had significant gender differences across the lifespan, including male-predominant Oatp1a1 and female-predominant Oatp1a4, Oatp2b1, Oct1, and Ent1. Oatp1a1 mRNA was more than 300% higher in male than female mice. In contrast, Oatp1a4 mRNA was more than 300% higher in female than male mice. The mRNAs of Oatp2b1, Oct1, and Ent1 were all twice as high in female as male mice.

**Phase−I Xenobiotic Metabolizing Enzymes during Aging.** With aging, the
mRNAs of Cyp1a1 (M and F) and Cyp1a2 (M and F) decreased approximately 50% between 3 and 27 months of age (Fig. 2). Cyp3a11 mRNA decreased about 50% between 3 and 27 months in female mice, whereas it remained relatively constant with age in male mice. Interestingly, Nqo1 mRNA remained relatively constant with age in male mice, but doubled between 3 and 27 months in female mice. The mRNAs of Cyp2b10, Cyp4a14, and Por remained relatively constant with aging in both genders. Most Cyps had female-predominant mRNA patterns across the lifespan: the mRNAs of Cyp2b10 (10-fold) and Cyp4a14 (8-fold) were higher in female than male mice. The mRNAs of Cyp1a1, Por, and Nqo1 were also higher in female mice. Cyp3a11 was the only Cyp gene with a male-predominant mRNA pattern.

The Fmo mRNAs did not change much with aging (Fig. 3). The mRNAs of Fmo2, 3, and 4 had a moderate increase between 3 and 27 months in female mice. Fmo4 mRNA in male mice also increased moderately between 3 and 27 months of age. The mRNAs of a number of Fmos had a female-predominant pattern across the lifespan. Remarkably, Fmo3 mRNA was about 200-fold higher in female than male mice. In addition, higher mRNAs in females were also observed for Fmo1, Fmo2, and Fmo4. In contrast, Fmo5 mRNA was male-predominant across the life span.

Adh4 mRNA decreased about 50% between 3 and 27 months of age in female mice (Fig. 4). Interestingly, Adh6b decreased 29% in male mice and increased 16% in female mice between 3 and 27 months. The mRNA of Adh7 increased 93% in female mice between 3 and 18 months. The mRNAs of Adh1, Adh5, and Adhfe1 did not change much with aging. Most Adh mRNAs did not show a consistent gender-divergent pattern across the lifespan. Adh4 mRNA was male-predominant, whereas Adh6b was...
female-predominant after one year old.

Aldh1a1 mRNA at 6-27 months was 33% lower than 3 months in female mice (Fig. 5). The mRNA of Aldh1a7 increased between 3 and 15 months of age in male mice. The mRNAs of Aldh2 and Aldh9a1 increased between 3 and 12 months and decreased thereafter in both genders. The mRNAs of the remaining Aldhs remained relatively constant with aging. A number of the Aldh mRNAs had a female-predominant pattern across the lifespan, including Aldh3a2, 4a1, and 6a1, which were 100% higher in female than male mice. Interestingly, Aldh7a1 mRNA was higher in male than female mice only between 15 and 27 months.

For many Cess, such as Ces1c, Ces1e, and Esd, the highest mRNAs were observed when the mice were about 12 months of age and decreased thereafter (Fig. 6). Decreased mRNAs were observed for Ces1f (F: 49%), Ces2c (M: 60%; F: 49%), and Ces3a (F: 75%) between 3 and 27 months of age. Most of the Ces and Pon mRNAs did not show gender-divergent patterns, except Ces2c and Ces3a, which had a male-predominant mRNA pattern, which was 200% higher in male than female mice.

**Phase-II Xenobiotic Metabolizing Enzymes during Aging.** Sults catalyze the conjugation of xenobiotics with sulfate groups, and the 3'-phosphoadenosine 5'-phosphosulfate synthetase (Papss) catalyzes the synthesis of the sulfate donor for all Sults. In contrast to the mRNA changes of most XPGs during aging, the mRNAs of a number Sults increased with age in mice (Fig. 7). Increased mRNAs with age were observed for Sult1a1 (M: 123%; F: 40%), Sult1e1 (M: 9.8-fold; F: 315-fold), Sult2a1/2 (F: 732%), and Papss1 (M: 211%, F: 209%). In contrast, Sult3a1 mRNA decreased 76% in the aged female mice, and Sult5a1 decreased about 50% between 3 and 27 months of
The mRNAs of Sult1d1 and Papss2 peaked at 18 months of age in female mice. The mRNAs of the majority of Sults had marked female-predominant patterns across the lifespan, namely Sult1a1, 1d1, 1e1, 2a1/2, 3a1, and 5a1. In addition, Papss2 mRNA had a female-predominant pattern.

Ugts catalyze the conjugation reaction with glucuronic acids, and UDP-glucose pyrophosphorylase 2 (Ugp2) catalyzes the synthesis of UDP-glucose from glucose-1-phosphate, which is an important precursor for the glucuronidation co-substrate UDP-glucuronic acid. The mRNAs of Ugt2b1 (M: 36%; F: 60%) and Ugt2b35 (M: 30%) markedly decreased between 3 and 27 months of age (Fig. 8). Ugt2b36 mRNA also decreased in aging male and female mice. Ugt1a6a mRNA reached peaks at 12 months of age in both genders. The mRNAs of Ugt3a1 and Ugt3a2 reached peaks at 12 months in male mice. Ugp2 mRNA decreased between mid-age and old-age in both genders. The mRNAs of Ugt1a1, 1a5, 1a9, 2a3, and 2b34 did not change much with aging. The mRNAs of some Ugts had female-predominant patterns across the lifespan, namely Ugt1a1, Ugt1a5, Ugt1a9, and Ugt2b34. In contrast, Ugt1a6a, 2b1, and 2b35 had male-predominant pattern.

Gsta1 mRNA increased markedly (M: 183%; F: 124%) between 3 and 27 months of age in both genders (Fig. 9). Gstm3 mRNA increased 112% between 3 and 27 months in female mice. A 35-50% decrease in mRNAs between 3 and 27 months was observed for Gstm4 (M), Gstm6 (F), Gstp1 (M), and Gstt2 (M and F). The mRNA of Gstp2 increased 95% between 3 and 27 months in male mice, whereas it decreased 43% in female mice. Mgst3 mRNA increased slightly between 6 and 18 months in both genders. Some of the Gst mRNAs had female-predominant patterns across the
lifespan (namely Gstm2, Gstt1, and Mgst3), whereas some had male-predominant patterns (namely Gstm6, Gstp1, and Gstp2). Gstp1 and Gstp2 mRNAs were about 300% higher in male than female mice, whereas Gstt1 and Mgst3 mRNAs were 200% higher in female than male mice.

Nat1 mRNA increased (M: 60%; F: 35%) between 3 and 27 months of age, whereas Nat2 mRNA did not change much with aging (Fig. 10). Comt mRNA decreased markedly (93%) between 3 and 27 months of age in female mice, whereas it remained relatively constant in male mice. The mRNAs of Nats did not have marked gender-divergent patterns across the lifespan. Comt mRNA had a male-predominant pattern in aging mice.

Efflux Transporters during Aging. Mrp4 mRNA increased 363% between 3 and 27 months of age in female mice, whereas it remained relatively constant in male mice (Fig. 11). A significant decrease of mRNA between 3 and 27 months was observed in male mice for Mrp3 (26%) and Bcrp (27%). Mrp2, Mrp3, Mrp6, Abca1, and Abcg8 mRNAs peaked at 12 months of age in female mice. The mRNAs of some efflux transporters had female-predominant mRNA patterns, such as Mrp3, Mrp4, Mate1, and Mdr2. In contrast, Bcrp had a male-predominant mRNA pattern.

Transcription factors during Aging. With aging, the mRNAs of all the transcription factors remained relatively constant (Fig. 12). The mRNAs of AhR and HNF1α were slightly higher at 24 and 27 months of age than at 3 months in male mice. The mRNAs of AhR and CAR were female-predominant across the life span. The mRNAs of remaining transcription factors did not have gender differences.

Hierarchical Cluster Analysis of Age-dependent mRNA Profiles of Xenobiotic
Processing Genes. The mRNAs of 44% of the 101 XPGs changed with aging in male mice and 63% changed in female mice. The mRNA profiles of these XPGs with age (Fig. 13) were analyzed by hierarchical cluster analysis, with red color representing higher and blue for lower mRNA abundance. The age-dependent mRNA changes of these XPGs could be classified into three clusters for both genders (Table 1), namely early-age pattern (highest level observed at 3-9 months of age), mid-age pattern (highest level observed at 12-18 months of age), and old-age pattern (highest level observed at 21-27 months of age). In male mice, mRNAs of 40 XPGs (e.g. Oatp1a1, Ces2c, Gstm4, Gstp1, and Ces1e) were lower in aged mice (over 21 months of age), whereas mRNAs of 4 XPGs (e.g. Oat2 and Gstm2) were higher in aged mice. In female mice, mRNAs of 43 XPGs (e.g. Oatp1a1, Cyp1a2, Ces1f, Sult3a1, Gstt2, Comt, Ent1, Fmo3, and Mrp6) were lower in aged mice, whereas mRNAs of 21 XPGs (e.g. Oatp1a4, Nqo1, Adh7, Sult2a1/2, Gsta1, and Mrp4) were higher in aged mice. In conclusion, the mRNAs of 40% of the XPGs were lower in aged male mice and 43% were lower in aged female mice.
DISCUSSION

The present study characterizes comprehensive mRNA profiles of XPGs with aging in livers of mice. Nine groups of mice from 3 to 27 months of age are examined, which gives much more details of age-dependent changes than previous reports (Peng et al., 2005; Mori et al., 2007; Lee et al., 2008). Using both male and female mice, the present study uniquely reveals gender-divergent mRNA profiles of XPGs across the lifespan.

Many uptake transporters are altered during aging. Oatps are sodium-independent uptake transporters whose substrates are diverse, mainly amphipathic organic compounds, including bile acids, hormones and their conjugates, toxins, and various drugs (Hagenbuch and Gui, 2008). The mRNA of liver-enriched Oatp1a1 decreases more than 50% in aged males, and surprisingly decreases almost 100% in aged females (Fig. 1). The mRNA of liver-specific Oatp1b2 decreases from 12 to 27 months of age in females. The mRNA of organic cation uptake transporter Oct1 decreases during aging in both genders. Taken together, liver appears to remove xenobiotics from the blood more slowly in the elderly.

The phase-I enzymes that are altered most during aging are Cyp and Ces families. Cyps are heme-containing enzymes, which catalyze monooxygenase reactions. The Cyp1, Cyp2, and Cyp3 families are the Cyps primarily involved in the metabolism of drugs, xenobiotics, and steroids (Monostory and Dvorak, 2011). Most of the Cyps quantified in the present study have an age-dependent decrease in mRNAs, markedly for Cyp1a1 (F) and Cyp1a2 (M and F), and to a lesser degree for Cyp3a11 (F) (Fig. 2). This suggests a possible mechanism for decreased metabolism of some xenobiotics in
the elderly. Cess catalyze the hydrolysis of many clinically useful drugs with ester moieties, resulting in mainly inactivation of drugs (such as heroin, cocaine, and flumazenil) as well as the activation of prodrugs (such as anticancer drugs CPT-11 and capecitabine) (Redinbo and Potter, 2005). The nomenclature of mouse Cess in the present study was reported previously (Holmes et al., 2010). The decreased mRNAs of Cess1f, 2c, and 3a during aging in females (Fig. 6) indicates it may take longer for the anticancer prodrugs to take effect in the elderly, and the ester drugs may have longer half-life in the elderly. For other phase-I enzymes, such as Fmos, Adhs, Aldhs, and Pons (Figs 3-6), aging has little effect on their mRNAs.

Aging has profound effects on altering the mRNAs of phase-II enzymes. All of the Sults quantified in the present study remain female-predominant during aging. The mRNAs of Sult1a1 and Papss1 increase during aging in both genders. Sult1e1 mRNA increases 9.8-fold in males and 315-fold in females between 3 and 27 months of age (Fig. 7). Sult1e1 sulfonates a variety of estrogens (Falany et al., 1995), and is the predominant determinant of the ratio of the active unconjugated estrogen to the inactive estrogen sulfate. In addition to the aging-induced decline of ovarian function and female sex hormone secretion, the increased Sult1e1 and possibly increased inactivation of estrogens might also contribute to decreased biologically active estrogens in aged females. The mRNA of Sult2a1/2 is abundant in females, but extremely low in males at young adulthood (3-month old), which is consistent with our previous publication (Alnouti and Klaassen, 2006). The DHEA-sulfotransferase Sult2A is known as a rat senescence marker protein, the expression of which is increased in aged male rats (Echchgadda et al., 2004). The present study shows that Sult2a1/2
mRNA increases 732% in aged female, but remains at extremely low levels in males during aging (Fig. 7). This discrepancy may result from species differences. Additional work is necessary to definitely determine the mechanisms underlying the age- and sex-dependent regulation of Sult2a1/2 in mice. In contrast to the markedly elevated mRNAs of Sults1a1, 1e1, and 2a1/2, Sult3a1 mRNA decreases 76% in the aged female mice. Sult3a1 is the only Sult isozyme that catalyzes \textit{N}-sulfonation, rather than \textit{O}-sulfonation of amines such as phenyltetrahydropyridine, aniline, 4-chloroaniline, 2-naphthylamine, and desipramine (Yoshinari et al., 1998). The 4-chloroaniline is carcinogenic in male rats and mice (Chhabra et al., 1991), mainly because of the toxic intermediate \textit{N}-phenylhydroxylamine. The C-hydroxylated product of 4-chloroaniline is sulfated and ready for excretion. The decreased mRNA of Sult3a1 after 12 months of age in male mice (Fig. 7) suggests that the detoxification of 4-chloroaniline might decline with age, which could favor cancer development by 4-chloroaniline.

The mRNAs of most Ugts remain relatively constant with aging (Fig. 8). However, Ugt2b1 mRNA decreases between 3 and 27 months of age in both genders. A previous study (Buckley and Klaassen, 2009) reported that the regulation of Ugt2b1 expression in mouse liver was attributed to male-pattern growth hormone secretion. This may explain the dramatic decrease of Ugt2b1 for male mice with age, and less dramatic for females.

Gsts catalyze the biotransformation and disposition of a wide range of chemical carcinogens, therapeutic drugs, the products of oxidative stress, and steroid hormones such as \textit{\Delta}5-androstenedione (Johansson and Mannervik, 2001). Gstp1 mRNA decreases markedly with age (Fig. 9). Gstp2 mRNA increases with age, but is
expressed at a much lower level than Gstp1. Gstp catalyzes the glutathione conjugation of cisplatin as a detoxification pathway (Townsend et al., 2009). The decrease in Gstp1 mRNA with aging in the present study provides a possible mechanism of changes in cisplatin toxicity during aging.

The Comt catalyzes the methylation of catecholamine neurotransmitters, L-DOPA, catecholestrogens (2- and 4-hydroxylated estrogen), as well as drugs, such as carbidopa and dobutamine. The highest Comt activity has been found in liver and kidney. Comt activity is also detected in several glands, muscle, adipose, blood cells and other tissues. One important function of Comt is to detoxify catecholestrogens, which appears to be important in initiating some estrogen-dependent cancers, possibly by generating reactive oxygen species and subsequent DNA damage (Weisz et al., 1998). The decreased Comt with aging in females (Fig. 10) suggest the detoxification of catecholestrogens in livers might decrease in aged females.

Most efflux transporters are altered during aging. The mRNAs of Mrp3 and Bcrp decrease during aging in both genders (Fig. 10). Mrp3 is important in transporting bilirubin glucuronides and bile acids from liver into blood, whereas Bcrp is important in liver for transporting sulfate and glucuronide conjugates of xenobiotics into bile. The decreased expression of Mrp3 and Bcrp with age suggests that glucuronide conjugates of xenobiotics as well as bilirubin might accumulate in hepatocytes with age. Interestingly, Mrp4 mRNA increases between 3 and 27 months of age in both genders, but more markedly in female mice (Fig. 10). A previous study shows higher mRNA of Mrp4 in female kidneys is due to repression by both 5alpha-dihydroxytestosterone and male-pattern growth hormone secretion in males (Maher et al., 2006). Hypothesizing
that similar mechanisms of hormonal regulation of female-predominant expression of Mrp4 exist in liver, the markedly elevated Mrp4 mRNA during aging possibly results from the decline of sex hormones and growth hormones with senescence.

All of the data collected in the present study regarding the expression of XPGs are at the mRNA level. One needs to be cautious when interpreting the data, because mRNA levels don’t always correlate with protein levels and protein activities. According to previous reports that the decreased protein contents (Peng et al., 2005; Mori et al., 2007) as well as enzyme activities (Warrington et al., 2004) of many phase-I and phase-II enzymes are regulated at the transcription level, the mRNA results from this study will provide a good indication for most enzymes and transporters. For transporters, it is especially difficult to quantify protein and activity of transporters, due to the lack of specific antibodies as well as specific substrates for activity assays.

In summary, the current study investigates the effect of aging on the mRNA changes of 101 xenobiotic processing genes (XPGs) in livers of male and female mice. Gender differences across the lifespan (significant at five ages or more) are observed for 52 XPGs, including 15 male-predominant (e.g. Oatp1a1, Cyp3a11, Ugt1a6a, Comt, and Bcrp) and 37 female-predominant genes (e.g. Oatp1a4, Cyp2b10, Sult1a1, Ugt1a1, and Mrp3). The mRNAs of 44% of the XPGs change with aging in male mice and 63% change in female mice. In male mice, mRNAs of 40 XPGs (e.g. Oatp1a1, Ces2c, Gstm4, Gstp1, and Ces1e) are lower in aged mice (over 21 months of age), whereas mRNAs of 4 XPGs (e.g. Oat2 and Gstm2) are higher in aged mice. In female mice, mRNAs of 43 XPGs (e.g. Oatp1a1, Cyp1a2, Ces1f, Sult3a1, Gstt2, Comt, Ent1, Fmo3, and Mrp6) are lower in aged mice, whereas mRNAs of 21 XPGs (e.g. Oatp1a4, Nqo1,
Adh7, Sult2a1/2, Gsta1, and Mrp4) are higher in aged mice. In conclusion, 51% of the 101 XPGs have gender differences in liver mRNAs across the lifespan of mice, and the mRNAs of 40% of the XPGs are lower in aged male mice and 43% are lower in aged female mice. Considering the role of XPGs in detoxification and elimination of drugs and environmental chemicals, the present results may improve the interpretation of long-term toxicity studies in old animals, and aid in determining the effective and safe dose for the elderly.
ACKNOWLEDGEMENTS

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Authorship Contributions

- Participated in research design- Fu, Csanaky, and Klaassen;
- Conducted experiments- Fu and Csanaky;
- Contributed new reagents or analytic tools- Fu and Klaassen;
- Performed data analysis- Fu and Klaassen;
- Write the manuscript- Fu, Csanaky, and Klaassen.
REFERENCES


Klaassen CD and Slitt AL (2005) Regulation of hepatic transporters by xenobiotic...


Peng FC, Jian WC, and Edwards RJ (2005) Profile of territrem metabolism and...


Wauthier V, Verbeeck RK, and Buc Calderon P (2004) Age-related changes in the protein and mRNA levels of CYP2E1 and CYP3A isoforms as well as in their...


Footnotes

a) This study was supported by the National Institute of Health [Grants ES-009649 and DK-081461].

b) The work was presented at the Annual Meeting of Society of Toxicology in 2009.

c) Please send reprint requests to Curtis D. Klaassen, Ph.D. E-mail: cklaasse@kumc.edu
Figure Legends

**Fig. 1** The mRNA profiles of uptake transporters with aging in livers of male and female mice. Asterisks (*) represent gender differences, determined by student t-test ($p<0.05$). Daggers (†) and double daggers (‡) represent age differences ($p<0.05$) compared to 3 months of age in male and female mice, respectively, determined by one-way ANOVA, followed by Duncan's post-hoc test.

**Fig. 2** The mRNA profiles of major cytochrome P450s (Cyps), P450 reductase (Por), and NAD(P)H:quinone oxidoreductase (Nqo1) with aging in livers of male and female mice. Asterisks (*) represent gender differences, determined by student t-test ($p<0.05$). Daggers (†) and double daggers (‡) represent age differences ($p<0.05$) compared to 3 months of age in male and female mice, respectively, determined by one-way ANOVA, followed by Duncan's post-hoc test.

**Fig. 3** The mRNA profiles of flavin-containing monooxygenases (Fmos) with aging in livers of male and female mice. Asterisks (*) represent gender differences, determined by student t-test ($p<0.05$). Daggers (†) and double daggers (‡) represent age differences ($p<0.05$) compared to 3 months of age in male and female mice, respectively, determined by one-way ANOVA, followed by Duncan's post-hoc test.

**Fig. 4** The mRNA profiles of alcohol dehydrogenases (Adhs) with aging in livers of male and female mice. Asterisks (*) represent gender differences, determined by student t-test ($p<0.05$). Daggers (†) and double daggers (‡) represent age differences ($p<0.05$) compared to 3 months of age in male and female mice, respectively, determined by one-way ANOVA, followed by Duncan's post-hoc test.

**Fig. 5** The mRNA profiles of aldehyde dehydrogenases (Aldhs) with aging in livers of
male and female mice. Asterisks (*) represent gender differences, determined by student t-test ($p<0.05$). Daggers (†) and double daggers (‡) represent age differences ($p<0.05$) compared to 3 months of age in male and female mice, respectively, determined by one-way ANOVA, followed by Duncan's post-hoc test.

**Fig. 6** The mRNA profiles of carboxylesterases (Cess) and paraoxonases (Pons) with aging in livers of male and female mice. Asterisks (*) represent gender differences, determined by student t-test ($p<0.05$). Daggers (†) and double daggers (‡) represent age differences ($p<0.05$) compared to 3 months of age in male and female mice, respectively, determined by one-way ANOVA, followed by Duncan's post-hoc test.

**Fig. 7** The mRNA profiles of sulfotransferases (Sults) and 3'-phosphoadenosine 5'-phosphosulfate synthetase (Papss) enzymes with aging in livers of male and female mice. Asterisks (*) represent gender differences, determined by student t-test ($p<0.05$). Daggers (†) and double daggers (‡) represent age differences ($p<0.05$) compared to 3 months of age in male and female mice, respectively, determined by one-way ANOVA, followed by Duncan's post-hoc test.

**Fig. 8** The mRNA profiles of UDP-glucuronosyltransferase (Ugts) and UDP-glucose pyrophosphorylase (Ugp2) with aging in livers of male and female mice. Asterisks (*) represent gender differences, determined by student t-test ($p<0.05$). Daggers (†) and double daggers (‡) represent age differences ($p<0.05$) compared to 3 months of age in male and female mice, respectively, determined by one-way ANOVA, followed by Duncan's post-hoc test.

**Fig. 9** The mRNA profiles of glutathione S-transferases (Gsts) with aging in livers of male and female mice. Asterisks (*) represent gender differences, determined by
student t-test ($p<0.05$). Daggers (†) and double daggers (‡) represent age differences ($p<0.05$) compared to 3 months of age in male and female mice, respectively, determined by one-way ANOVA, followed by Duncan's post-hoc test.

**Fig. 10** The mRNA profiles of $N$-acetyltransferases (Nats) and catechol-$O$-methyl transferase (Comt) with aging in livers of male and female mice. Asterisks (*) represent gender differences, determined by student t-test ($p<0.05$). Daggers (†) and double daggers (‡) represent age differences ($p<0.05$) compared to 3 months of age in male and female mice, respectively, determined by one-way ANOVA, followed by Duncan's post-hoc test.

**Fig. 11** The mRNA profiles of efflux transporters with aging in livers of male and female mice. Asterisks (*) represent gender differences, determined by student t-test ($p<0.05$). Daggers (†) and double daggers (‡) represent age differences ($p<0.05$) compared to 3 months of age in male and female mice, respectively, determined by one-way ANOVA, followed by Duncan's post-hoc test.

**Fig. 12** The mRNA profiles of major transcription factors with aging in livers of male and female mice. Asterisks (*) represent gender differences, determined by student t-test ($p<0.05$). Daggers (†) and double daggers (‡) represent age differences ($p<0.05$) compared to 3 months of age in male and female mice, respectively, determined by one-way ANOVA, followed by Duncan's post-hoc test.

**Fig. 13** Hierarchical cluster analysis of the mRNA profiles of XPGs during aging in livers of male and female mice. Clustering analysis results are shown in the dendrogram, with y-axis representing the XPGs that change with aging and x-axis representing the nine age groups of mice (from 3 to 27 months). The red color
DMD #44461

represents higher mRNA abundance and blue represents lower mRNA abundance. The spectrum of each gene is standardized and specific to the scale of its own mRNA. Therefore, it is not valid to compare the mRNAs among different genes according to the color.
Table 1. Summary of three patterns of age-dependent mRNA changes of XPGs

<table>
<thead>
<tr>
<th>Patterns</th>
<th>Male</th>
<th>Female</th>
</tr>
</thead>
<tbody>
<tr>
<td>early-age</td>
<td>15/101*</td>
<td>20/101</td>
</tr>
<tr>
<td>mid-age</td>
<td>25/101</td>
<td>23/101</td>
</tr>
<tr>
<td>old-age</td>
<td>4/101</td>
<td>21/101</td>
</tr>
</tbody>
</table>

XPGs that don't change with aging:
* means the ratio of the number of XPGs in each age-dependent mRNA change pattern to the total number of XPGs.
Fig. 2

Comparison of mRNA levels of various enzymes across different ages and genders:

- **Cyp1a1**
- **Cyp1a2**
- **Cyp2b10**
- **Cyp3a11**
- **Cyp4a14**
- **Por**
- **Nqo1**

*Note: Data points marked with an asterisk (*) indicate significant differences.*
Fig. 3

- **Fmo1**: mRNA levels normalized to Gapdh for different ages grouped by sex.
- **Fmo2**: Similar to Fmo1, but data for both male and female are shown.
- **Fmo3**, **Fmo4**, **Fmo5**, **Fmo9**, **Fmo12**, and **Fmo13**: Similar trends with varying levels of expression over time.

Axes:
- **Y-axis**: mRNA levels (normalized to Gapdh).
- **X-axis**: Age (months).

Legend:
- *: Statistically significant.
- †: Other significant marker.
- Male and Female data distinguished by symbols.

The graphs illustrate the fluctuation in mRNA levels for different FMOD enzymes across various age groups, with significant markers indicated for each.
Fig. 6

![Graph showing mRNA levels normalized to Gapdh for different enzymes with age (months) for both male and female.]
Fig. 7

[Graph showing mRNA (normalized to Gapdh) levels for Sult1a1, Sult1d1, Sult1e1, Sult2a1/2, Sult3a1, Sult5a1, Papss1, and Papss2 over age (months)].
Fig. 8

The figure shows the mRNA expression levels of various genes normalized to Gapdh across different age groups. The graphs represent gene expression over age (months) and are color-coded by gender (black for male, white for female). Significant differences are indicated by asterisks (*), with double asterisks denoting higher significance levels.

- **Ugt1a1**: Levels remain relatively constant with minor fluctuations.
- **Ugt1a5**: Shows more variation, with peaks and troughs at different ages.
- **Ugt1a6a**: Consistently low with minimal change.
- **Ugt1a9**: Similar to Ugt1a6a, with slight variations.
- **Ugt2a3**: Demonstrates pronounced peaks and troughs at specified ages.
- **Ugt2b1**: Exhibits distinct patterns, with notable peaks and declines.
- **Ut2b34**: Shows a steady increase and decrease pattern.
- **Ut2b35**: Features a more gradual increase and decrease.
- **Ugt2b36**: Exhibits a steady pattern with minor fluctuations.
- **Ugt3a1**: Displays a sharp peak and fall off.
- **Ugt3a2**: Consistently low, with minimal variation.
- **Ugp2**: Shows a steady pattern with slight peaks and troughs.

Significance levels are indicated by asterisks (*), with double asterisks denoting higher significance levels.
Fig. 10

<table>
<thead>
<tr>
<th>mRNA (normalized to Gapdh)</th>
<th>Nat1</th>
<th>Nat2</th>
</tr>
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<tbody>
<tr>
<td>RLU/10 μg RNA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age (months)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Nat1**

- Male: *
- Female: *

**Nat2**

- Male: *
- Female: *

**Comt**

- Male: *
- Female: *
Fig. 11

![Graph showing mRNA levels of various transporters over age (months).](image-url)

- **Mrp2**: Male (solid line) and female (dotted line) data with asterisks indicating significant differences.
- **Mrp3**: Male (solid line) and female (dotted line) data with asterisks and a dagger indicating significant differences.
- **Mrp4**: Male (solid line) and female (dotted line) data with asterisks indicating significant differences.
- **Mrp6**: Male (solid line) and female (dotted line) data with asterisks indicating significant differences.
- **Mate1**: Male (solid line) and female (dotted line) data with asterisks indicating significant differences.
- **Mdr2**: Male (solid line) and female (dotted line) data with asterisks indicating significant differences.
- **Bcrp**: Male (solid line) and female (dotted line) data with asterisks indicating significant differences.
- **Abca1**: Male (solid line) and female (dotted line) data with asterisks indicating significant differences.
- **Abcg5**: Male (solid line) and female (dotted line) data with asterisks indicating significant differences.
- **Abcg8**: Male (solid line) and female (dotted line) data with asterisks indicating significant differences.

* mRNA (normalized to Gapdh) vs. Age (months)
Fig. 12

mRNA (normalized to Gapdh) vs. Age (months)

- AhR
- CAR
- PXR
- PPARα
- RXRα
- HNF1α
- HNF4α

Significance levels:
- * p < 0.05
- ** p < 0.01
- † p < 0.01 (female vs. male)
Supplemental Fig. 1  The real-time qRT-PCR validates that Gapdh mRNA did not have sex- or age-differences. Total RNA was extracted from livers of male and female mice at 3 and 27 months of age. The mRNA of the Gapdh was quantified by real-time qRT-PCR and the cycle threshold value was shown. The primer sequences for GAPDH (NM_008084.2) were 5'-aactttggcattgtggaagg-3' (forward) and 5’-ggatgcagggatgttct-3' (reverse). There were not differences between sex or age of Gapdh mRNA.
Supplemental Material

Article title: Effect of Aging on mRNA Profiles of Drug Metabolizing Enzymes and Transporters in Livers of Male and Female Mice

Article authors: Zidong Donna Fu, Iván L. Csanaky, and Curtis D. Klaassen

Journal Drug Metabolism and Disposition

Supplementary Table 1. Oligonucleotide probes for quantifying mouse Comt (Accession No: NM_001111063) mRNA by the branched DNA assay

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• CE, capture extender; LE, label extender; BL, blocker.