Age-associated changes of cytochrome P450 and related phase-2 gene/proteins in livers of rats

Shangfu Xu [1] , **Anling Hu** [1] , **Lu Xie** [1] , **Jiajia Liu** [1] , **Qin Wu** [1] , **Jie J Liu** [Corresp. 1]

[1] Key Lab for Basic Pharmacology of Ministry of Education, Zunyi Medical University, Zunyi, China

Corresponding Author: Jie J Liu
Email address: Jie@liuonline.com

Cytochrome P450s (CYPs) are phase-I metabolic enzymes playing important roles in drug metabolism, dietary chemicals and endogenous molecules. Age is a key factor influencing P450s expression. Thus, age-related changes of CYP 1-4 families and bile acid homeostasis-related CYPs, the corresponding nuclear receptors and a few phase-II genes were examined. Livers from male Sprague-Dawley rats at fetus (-2 d), neonates (1, 7, and 14 d), weaning (21 d), puberty (28 and 35 d), adulthood (60 and 180 d), and aging (540 and 800 d) were collected and subjected to qPCR analysis. Liver proteins from 14, 28, 60, 180, 540 and 800 days of age were also extracted for selected protein analysis by Western-blot. In general, there were three patterns of their expression: Some of the drug-metabolizing enzymes and related nuclear receptors were low in fetal and neonatal stage, increased with liver maturation and decreased quickly at aging (AhR, Cyp1a1, Cyp2b1, Cyp2b2, Cyp3a1, Cyp3a2, Ugt1a2); the majority of P450s (Cyp1a2, Cyp2c6, Cyp2c11, Cyp2d2, Cyp2e1, CAR, PXR, FXR, Cyp7a1, Cyp7b1. Cyp8b1, Cyp27a1, Ugt1a1, Sult1a1, Sult1a2) maintained relatively high levels throughout the adulthood, and decreased at 800 days of age; and some had an early peak between 7 and 14 days (CAR, PXR, PPARα, Cyp4a1, Ugt1a2). The protein expression of CYP1A2, CYP2B1, CYP2E1, CYP3A1, CYP4A1, and CYP7A1 corresponded the trend of mRNA changes. In summary, this study characterized three expression patterns of 16 CYPs, 5 nuclear receptors, and 4 phase-II genes during development and aging in rat liver, adding to our understanding of age-related CYP expression changes and age-related disorders.

1 **Title page for PeerJ**

2

3 **Age-associated changes of cytochrome P450 and related phase-2 gene/proteins**

4 **in livers of rats**

5

6 Shang-Fu Xu, An-Ling Hu, Lu Xie, Jia-Jia Liu, Qin Wu and Jie Liu[*]

7

8 Key Lab for Basic Pharmacology of Ministry of Education and Joint International

9 Research Laboratory of Ethnomedicine, Zunyi Medical University, Zunyi, China;

10

11 Running Title Page

12 Title: **Age-associated changes of P450 in livers of rats**

13 Corresponding author:

14 Jie Liu, Jie@liuonline.com; or Jieliu@zmc.edu.cn

15 Key Lab for Basic Pharmacology of Ministry of Education and Joint International

16 Research Laboratory of Ethnomedicine, Zunyi Medical University, Zunyi, China;

17

18 **Abbreviations:**

19 AhR, aryl hydrocarbon receptor; BA, Bile acid; CAR, constitutive androstane receptor;

20 CYPs, cytochrome P450s; FXR, Farnesoid X receptor; NRs, nuclear receptors; PPAR,

21 peroxisome proliferator-activated receptor; PXR, pregnane X receptor (PXR); SHP,

22 small heterodimer partner; SULTs, Sulfotransferases; TCDD, tetrachlorodibenzodioxin;

23 UGTs, UDP-glucuronosyltransferases;

Highlights (review only):

25

26 • Livers of rats at -2, 1, 7, 14, 21, 28, 35, 60, 180, 540 and 800 days were

27   analyzed.

28 • A total of 16 cytochrome P450s, 5 nuclear receptors, 4 phase-II genes were

29   examined.

30 • Three patterns of age-associated gene expressions were characterized.

31 • Protein of CYP1A2, CYP2B1, CYP2E1, CYP3A1, CYP3A4, CYP4A1, and

32   CYP7A1 verified PCR.

33 • Age-associated P450 gene expressions in relation to diseases were discussed.

35   **ABSTRACT**

36   Cytochrome P450s (CYPs) are phase-I metabolic enzymes playing important roles in

37   drug metabolism, dietary chemicals and endogenous molecules. Age is a key factor

38   influencing P450s expression. Thus, age-related changes of CYP 1-4 families and bile

39   acid homeostasis-related CYPs, the corresponding nuclear receptors and a few phase-

40   II genes were examined. Livers from male Sprague-Dawley rats at fetus (-2 d),

41   neonates (1, 7, and 14 d), weanling (21 d), puberty (28 and 35 d), adulthood (60 and

42   180 d), and aging (540 and 800 d) were collected and subjected to qPCR analysis.

43   Liver proteins from 14, 28, 60, 180, 540 and 800 days of age were also extracted for

44   selected protein analysis by Western-blot. In general, there were three patterns of their

45   expression: Some of the drug-metabolizing enzymes and related nuclear receptors

46   were low in fetal and neonatal stage, increased with liver maturation and decreased

47   quickly at aging (AhR, Cyp1a1, Cyp2b1, Cyp2b2, Cyp3a1, Cyp3a2, Ugt1a2); the

48   majority of P450s (Cyp1a2, Cyp2c6, Cyp2c11, Cyp2d2, Cyp2e1, CAR, PXR, FXR,

49   Cyp7a1, Cyp7b1. Cyp8b1, Cyp27a1, Ugt1a1, Sult1a1, Sult1a2) maintained relatively

50   high levels throughout the adulthood, and decreased at 800 days of age; and some had

51   an early peak between 7 and 14 days (CAR, PXR, PPARα, Cyp4a1, Ugt1a2). The

52   protein expression of CYP1A2, CYP2B1, CYP2E1, CYP3A1, CYP4A1, and CYP7A1

53   corresponded the trend of mRNA changes. In summary, this study characterized three

54   expression patterns of 16 CYPs, 5 nuclear receptors, and 4 phase-II genes during

55   development and aging in rat liver, adding to our understanding of age-related CYP

56   expression changes and age-related disorders.

57   **Keywords:** Ontogeny; Aging; Cytochrome P450s; Nuclear receptors; Rat liver,

58   mRNA/protein expression.

Introduction

59

60   Cytochrome P450s (CYPs) are phase-I metabolic enzymes playing important roles

61   in drug metabolism, dietary chemicals, as well as endogenous molecules in the liver.

62   CYP1, 2, 3, and 4 families are responsible for the biotransformation of most foreign

63   substances including 70-80% of all drugs in clinical use, CYP 4 families also participate

64   in lipid metabolism.(Cui et al. 2012; Zanger & Schwab 2013) The CYP7 families,

65   together with CYP8 and CYP27 are important for cholesterol and bile acid metabolism

66   and homeostasis.(Cuesta de Juan et al. 2007; Liu et al. 2014)

67   CYPs are regulated by many physiological, genetic, environmental, and pathological

68   factors. For example, CYPs expression can be affected by hormones (Daskalopoulos et

69   al. 2012), cytokines (Kot & Daujat-Chavanieu 2018), pregnancy,(He et al. 2005) sex,

70   (Agrawal & Shapiro 2003; Das et al. 2014) and age. CYPs are subjected to age-

71   dependent changes in cell differentiation(Czekaj et al. 2010) and epigenetic

72   regulation,(Li et al. 2009) and age-related metabolic syndrome,(Bondarenko et al. 2016)

73   kidney diseases,(Velenosi et al. 2012) diabetes,(Park et al. 2016) nonalcoholic

74   steatohepatitis,(Li et al. 2017) virus hepatitis, and cirrhosis.(Kirby et al. 1996)

75   Age of animals greatly affects drug metabolism,(Durnas et al. 1990) alters

76   pharmacokinetics of xenobiotics,(Matalova et al. 2016; Shi & Klotz 2011) and thus alters

77   the sensitivity to drugs and toxicants such as acetaminophen,(Mach et al. 2014)

78   isoniazid,(Mach et al. 2016) aflatoxin B1,(Kirby et al. 1996; Wang et al. 2018) and

79   thioacetamide.(Kang et al. 2008) Age also influences drug-drug interactions.(Jia et al.

80   2014) Age-associated changes in P450 and corresponding nuclear factors are a major

81   determinant in CYP regulation of drug metabolism, especially during development

82 (children) and in senescence (elderly).(Durnas et al. 1990; Kilanowicz et al. 2015; Shi &

83 Klotz 2011)

84   The expression and maturation of CYPs during development is a major topic of

85 research,(Kilanowicz et al. 2015) and immature rats have been proposed as a potential

86 model for xenobiotics risk evaluation for children.(McPhail et al. 2016) The ontogeny of

87 CYPs greatly affects the drug metabolism especially during the developmental

88 period,(de Zwart et al. 2008) and is the major cause of altered susceptibility to drugs

89 and toxicants in children.(Li et al. 2017; Yun et al. 2010)

90   Aging is a physiological process characterized by progressive functional decline in

91 various organs over time. Aging is an important factor leading to alterations in the

92 biotransformation, either by reduced expression or decreased function. Many

93 cytochrome P450 genes from CYP 1-3 families show decreased expression in the older

94 rats.(Yun et al. 2010) The ability of liver CYPs to metabolize xenobiotics decreases with

95 aging *in vitro* (Salmin et al. 2017) and *in vivo,*(Wauthier et al. 2007) and hepatic CYP

96 mRNA expressions are decreased with aging.(Mori et al. 2007) The ability of CYPs in

97 response to inducers such as phenobarbital is also decreased in old rats.(Agrawal &

98 Shapiro 2003) Age-associated CYP3A expression changes in the liver are more

99 remarkable as compared to that occurred in the intestine and kidney, and are tissue-

100 specific.(Warrington et al. 2004) Since P450 enzymes in humans are regulated in a

101 manner similar to that in animals.(Durnas et al. 1990; Wauthier et al. 2007) Thus, to

102 examine CYP expressions in the old laboratory animals would help evaluation of drug

103 metabolism, efficacy and toxicity in the elderly.

104   In humans, three patterns of drug metabolizing enzymes are proposed.(Hines 2008)

105   The first pattern (e.g.,CYP3A7) is expressed at the highest level at their highest level

106   during the first trimester and either remains at high concentrations or decreases during

107   gestation, but is silenced or reduced within one to two years after birth; the 2nd pattern

108   (e.g.,SULT1A1) is expressed at relatively constant levels throughout gestation and

109   minimal changes are observed postnatally; and the 3rd pattern (e.g.ADH1C) is not

110   expressed or is expressed at low levels in the fetus.(Hines 2008; Hines 2013) Age-

111   associated changes of drug metabolism in humans, especially during stages before

112   birth and during early development (neonate/infant/child), could be studied in laboratory

113   animals.(Hines 2013; Saghir et al. 2012)

114   We have collected liver samples of Sprague Dawley (SD) rats from prenatal (-2 d),

115   neonatal (1, 7, and 14 d), weanling (21 d), puberty (28 and 35 d), adulthood (60 and 180

116   d), and aging (540 and 800 d), and have published several papers related to hepatic

117   uptake Oatp transporters(Hou et al. 2014) and hepatic efflux MRP transporters.(Zhu et

118   al. 2017) We have also used these samples to characterize the Nrf2 antioxidant

119   pathways,(Xu et al. 2018b) glutathione S-transferases,(Xu et al. 2018a) and the

120   antioxidant metallothionein gene expression.(Hou WY 2014)   The goals of the current

121   study were to use these samples to quantify the expression of 16 major CYP isoforms in

122   rat livers, 5 corresponding nuclear receptors (NRs), and 4 phase-II conjugation genes.

123   Protein expressions of selected CYPs were also performed to confirm qPCR results.

124   Similar to three patterns of CYP expression during mouse liver development,(Hart et al.

125   2009) the current study identified three patterns of CYP expression in the liver of rats at

126   11 time points of entire life span to help our understanding the age-associated changes

127   in these important phase-I and phase-II drug metabolism genes, and age-associated

128   disorders.

129

Materials and Methods

130

131   **Animals.** Adult male and female SD rats (250-300 g, 10 males and 30 females)

132   were purchased from the Experimental Animal Center of Third Military Medical

133   University (Chongqing, China; Certificate No: CXK 2007–0005). Rats were kept in a

134   SPF-grade animal Facilities with controlled environment (22 ± 1 °C, 50 ± 2% humidity

135   and a 12 h: 12 h light: dark cycle) at Key Lab for Basic Pharmacology of Ministry of

136   Education. Rats had free access to purified water and standard laboratory chow

137   (Experimental Animal Center, Chongqing, China). All animal care and experimental

138   protocols were complied with the Animal Management Guidelines of China and

139   approved by the Animal Use and Care Committee of Zunyi Medical University

140   (2012−02).

141   **Sample collection.** Rats were acclimatized for one week before timely mating

142   overnight and a positive vaginal plug next morning was considered as gestation day 1.

143   Livers of offspring male rats were collected at gestation day 19 (−2 d), at birth (1 d), at

144   the neonatal stage (7 and 14 d), at weanling (21 d), at puberty (28 and 35 d), at the

145   adulthood (60 and 180 d), and at aging (540 and 800 d). Six samples per time point

146   were collected, however, n=4-5 was used to for a 96-well qPCR plate to hold all time

147   points. Rats were anesthetized by chloral hydrate (10%, 5 mL/kg, ip), followed by

148   decapitation to minimize potential pain and distress. Liver tissues were stored at −80

149   °C prior to analysis.

150   **Real-time RT-PCR analysis.** Liver total RNA was extracted by using RNAiso

151   Plus kit (TaKaRa Biotechnology Co., Ltd., Dalian, China). The quality and quantity of

152   total RNA were determined by nanodrop and the 260/280 nm ratio > 1.8. The total RNA

153   was reverse transcribed to cDNA (Applied Biosystems, Foster City, CA) and real-time

154   RT-PCR analysis (Bio-Rad Laboratories, Hercules, CA) was conducted as described

155   (Xu et al. 2018b). Relative expression of genes was calculated by the $2^{-\Delta\Delta Ct}$ method and

156   normalized to the house-keeping gene β-actin or GAPDH (results were similar, data not

157   shown), and expressed as relative transcript levels, setting controls as 100%. The

158   primer sequences used in this study were shown in the Supplemental Table 1.

159   **Western-blot analysis.** Liver tissues (50-100 mg) were homogenized in RIPA

160   lysis buffer (Beyotime Institute of Biotechnology, Shanghai, China) containing 1 mM

161   phenylmethanesulfonyl fluoride (PMSF) and freshly prepared proteinase inhibitors.

162   Protein concentrations were quantified by the BCA assay (Beyotime Institute of

163   Biotechnology, Shanghai, China) and denatured (90 °C, 10 min with Nupage Loading

164   buffer). Aliquoted proteins (30 μg) were separated on NUPAGE 10% BT gels (Thermo

165   Fisher Scientific, Waltham, MA) and transferred to PVDF membranes. After blocking

166   with 5% nonfat milk at room temperature for two hours, membranes were incubated with

167   primary mouse antibody against β-actin, rabbit polyclonal antibodies against rat

168   CYP1A2 (bs-2589R), CYP2B1 (bs-14177R), CYP2E1 (bs-4562R), CYP3A1 (bs-

169   20586R), CYP4A1 (bs-5054R) and CYP7A1 (bs-21429R) (1:1000) (Biosynthesis

170   Biotechnology Co., LTD. Beijing, China) overnight at 4 °C. After washes with TBST,

171   membranes were incubated with horseradish peroxidase conjugated anti-rabbit, anti-

172   mouse IgG secondary antibodies (1:5000) for 1 h at room temperature. Protein antibody

173   complexes were visualized using an Enhanced Chemiluminescent reagent and a

174   ChemiDoc XRS system (Bio Rad Laboratories, Inc., USA). Band intensities were semi-

175   quantified by densitometry using Quantity One® software (version 4.6.2, Bio Rad

176   Laboratories, Inc., USA) (Xu et al. 2018a).

177   **Statistical analysis.** The software SPSS version 16.0 (SPSS, Inc., Chicago, IL,

178   USA) was used for statistical analysis. Data were expressed as the mean ± SEM (n= 4-

179   5 per time point). Age associated differences were analyzed by one-way analysis of

180   variance, followed by the least significant difference post hoc test, *p* < 0.05 was

181   considered to indicate a statistically significant difference from the levels of birth.

182

183   **Results**

184

185   ***Age-related expression of CYP-1 family***

186   The expression of CYP-1 family is shown in Figure 1. Aryl hydrocarbon receptor

187   (AhR) mainly mediates the expression of CYP1A1 and CYP1A2 proteins. 2,3,7,8-

188   Tetrachlorodibenzodioxin (TCDD) is a typical CYP1A1 inducer. When TCDD is

189   combined with AhR, AhR is dissociated from the complex and transferred to the nucleus.

190   It forms heteromeric dimers with AhR nuclear transport protein, and then induces the

191   expression of target genes. In this way, TCDD and other AhR activators significantly

192   induce the expression of CYP1A genes.(Aleksunes & Klaassen 2012) AhR was low in

193   fetal livers (-2d), and gradually increase after birth, and reached the highest levels at 35

194   days of age (6.7-fold of birth), and gradually declined, and at 800 days of age, it

195   remained 4.2-fold higher than at birth (Figure 1A). Cyp1a1 was low at -2 days of age

196   through 14 days of age, and begin to increase at 21 days of age, rapidly increased 48-

197   fold at 28-60 days of age, and decline rapidly after 60 days of age, and returned to 2.4-

198   fold of the birth level at 800 days of age (Figure 1B). In contrast, Cyp1a2 followed the

199 similar pattern as AhR. Cyp1a2 increased dramatically after birth, reached 250-fold at

200 weanling (21 day), and peaked on 35 days of age (1100 fold). It was gradually declined

201 afterwards. Similar to AhR, Cyp1a2 still remained at the high level at 800 days of age

202 (880 fold of birth). CYP1A2 protein expression followed the similar pattern (Figure 1C).

203

204 ***Age-related expression of CYP-2 family***

205 The expression of CYP-2 family is shown in Figure 2. Constitutive androstane

206 receptor (CAR) is a nuclear receptor of steroid hormones. It regulates the metabolizing

207 enzymes and transporters in liver and small intestine. CAR mediates endogenous

208 hormone or exogenous drug reactions, such as phenobarbital, and transcriptionally

209 regulates CYP2 expression.(Aleksunes & Klaassen 2012) CAR was low in fetal livers (-

210 2d), and gradually increase after birth, first peaked at 7 days of age, and reached the

211 highest levels at 60 days of age (6-fold of birth), and gradually declined, and at 800

212 days of age, it remained 2.3-fold higher than at birth (Figure 2A). Cyp2b2 was low in

213 fetal livers (-2 d), and begin to increase after birth, and rapidly increased at weaning (21

214 d), reaching the peak at 35 days of age (7-fold of birth) and decreased afterwards, and

215 returned to birth level after 540 days of age (Figure 2B). The expression of Cyp2b1

216 followed the similar pattern as Cyp2b2, and the expression of CYP2B1 protein followed

217 the similar pattern (Figure 2C). Cyp2c6 increased after weanling, reached the peak of

218 liver maturation (350 fold) at 60 days of age, and remained high till 540 days of age, and

219 decreased to 250-fold over birth levels at 800 days of age (Figure 2D). Cyp2c11

220 increased 800-fold at puberty (35 days of age), but dramatically increased with liver

221 maturation (60000-fold at 60 days, 98000-fold at 180 days, and 110000-fold at 540 days

222   of age), and rapidly decreased at aging of 800 days of age, but it was still 40000-fold

223   over the birth level. (Figure 2E). Cyp2d2 increased gradually after birth, reached the

224   peak (9-fold) of liver maturation at 35 -180 days of age, and decreased to 6-fold of birth

225   at 540 and 800 days of age (Figure 2F). The expression pattern of Cyp2e1 was

226   relatively stable: Cyp2e1 increased rapidly after birth, reached 30-fold of the birth levels

227   at weanling (21 days of age), and peaked on 35 days of age (35 fold). It was gradually

228   declined but remained at the high level at the age of 800 days (22 fold of the birth level).

229   CYP2E1 protein expression followed the similar pattern (Figure 2G).

230

231   ***Age-related mRNA expression of CYP-3 family***

232   The expression of CYP-3 family is shown in Figure 3. Pregnane X receptor (PXR)

233   is a highly conserved ligand-dependent transcription factor. It is mainly expressed in the

234   liver and partly expressed in the colon and small intestine. The regulation of CYP3A by

235   PXR-mediated signaling pathway is an important pathway of drug

236   metabolism.(Aleksunes & Klaassen 2012) The expression of PXR was relatively stable

237   throughout the life with approximately 2-fold variations (Figure 3A). The expression of

238   Cyp3a2 markedly increased 4 fold at 7 days of age, and rapidly increased after

239   weanling (21 days of age), reached the peak at 28 days of age (25-fold of birth) and

240   decreased gradually afterwards, and the level was still 5.4-fold of the birth level at 800

241   days of age (Figure 3B). Cyp3a1 follows similar pattern as Cyp3a2. It was markedly

242   increased after weanling, reached the peak at 28 days of age (13-fold of the birth level)

243   and decreased gradually afterwards, and the level was still 3.4-fold of the birth level at

244   800 days of age. The expression of Cyp3a1protein followed the similar patter (Figure

245   3C).

246

Age-related expression of CYP-4 family

248   The expression of CYP-4 family is shown in Figure 4. Peroxisome proliferator-

249   activated receptors (PPARs) nuclear receptor family regulates the expression of genes

250   that control fatty acid synthesis, storage, and catabolism. PPARs mainly include PPARα,

251   PPARβ and PPARγ. The activation of PPAR can improve insulin resistance, slow down

252   atherosclerosis, and promote the metabolism of cholesterol. PPARα regulates induction

253   of CYP4A gene.(Aleksunes & Klaassen 2012) The expression of PPARα was relatively

254   stable throughout the life, except for 7 and 14 days of age (2-fold of the birth level), and

255   at 800 days of age, its levels returned to the birth level (Figure 4A). Cyp4a1 started to

256   increase after birth, with the first peak at 7 days (3.5-fold) and decreased after 14 days

257   of age, and again gradually increased after weanling and remained high throughout 540

258   days of age. At 800 days of age, it was still 1.8-fold of the birth level. The expression of

259   CYP4A1 protein followed the similar pattern (Figure 4B).

260

Age-related mRNA expression of CYPs involved in bile acids homeostasis

262   The expression of CYPs involved in cholesterol and bile acids homeostasis is

263   shown in Figure 5. Bile acids (BAs) are the endogenous ligands of farnesoid X receptor

264   (FXR), so FXR is also called the BA receptor. Cholesterol 7α hydroxylase (Cyp7a1) is

265   important for BA synthesis. When BA overloads in the liver, toxicity to liver cells occurs,

266   including oxidative stress, inflammation, necrosis and even cirrhosis.(Cuesta de Juan et

267   al. 2007; Liu et al. 2014) The expression of FXR increased at 14 days of age (5 fold),

268   and gradually increased afterwards with age, reached peak at 180 days (13 fold), and

269   still high at 800 days (6.8 fold) (Figure 5A). Cyp7a1 started to increase at 14 days of

270   age, reached the peak at 28 days (37 fold of birth), and remained high throughout the

271   adulthood, and was still 11-fold higher than the birth level at 800 days of age. The

272   expression of CYP7A1 protein followed similar pattern (Figure 5B). The expression of

273   Cyp7b1 started to increase after birth, reached the peak at 28 days of age, and

274   decreased at 800 days of age (Figure 5C). Cyp8b1 increased at birth and decreased at

275   7 days of age. Cyp8b1 started to increase again after weanling, reached the peak at

276   adulthoods at 180 days of age, and gradually decreased thereafter. It was still higher at

277   800 days of age (Figure 5D). The expression of Cyp27a1 held a similar pattern,

278   reached the peak on 35 days of age (5.8-fold over the birth level), and remained the

279   high levels throughout 540 days of age, and decreased at 800 days of age with 3-fold

280   higher over the birth level (Figure 5E).

281

282   ***Age-related mRNA expression of UGT and SULT families***

283   The expression of UGT and SULT families is shown in Figure 6. UDP-

284   glucuronosyltransferases (UGTs) and sulfotransferases (SULTs) are the two most

285   important phase-2 conjugation enzymes to conjugate the CYP catalyzed metabolites

286   and drugs contain functional groups such as hydroxyls and carboxylic acids.(Coughtrie

287   2015) Glucuronidation involves the reaction of uridine 5′-diphosphoglucuronic acid with

288   a number of functional groups generated from CYP metabolism and is a major

289   mechanism for the formation of water-soluble substrates for their elimination in bile or in

290   urine, especially for the clearance of a number of drugs in children.(Krekels et al. 2012)

291   SULTs transfer the sulfuryl moiety from the universal donor PAPS (3'-

292   phosphoadenosine 5'-phosphosulfate) to a wide variety of substrates with hydroxyl- or

293   amino-groups after CYP metabolism.(Coughtrie 2016) Ugt1a1 was low in fetal livers (-2

294   d), and gradually increase after birth, marked increase after weanling (21 days), and

295   reached the highest levels at 28 days of age (7.8-fold of birth), and gradually declined,

296   and at 800 days of age, it remained 4-fold higher than the level of birth (Figure 6A). The

297   expression of Ugt1a2 was relatively stable throughout the life, except for a small peak at

298   day 28 and 35 days of age (2.5-fold higher than the birth level) (Figure 6B). The

299   expression of Sult1a1 started to increase at 14 days of age, reached the peak at 180

300   days of age (20-fold), remained 14-fold higher than the birth levels (Figure 6C). The

301   expression of Sult1a2 started to increase at 14 days of age, reached the first peak at

302   weanling (15 fold), but dramatically increased after 35 days of age, reached 110-fold at

303   60 days of age, and 190-fold at 540 days of age. It remained 70-fold higher than the

304   levels of birth (Figure 6D). The expression pattern of Sult1a1 was quite similar to the

305   expression pattern of Cyp2c11.

306

307

Discussion

308

309   The present study characterized age-related expression of 16 CYPs, 5 NRs, and 4

310   phase-II genes in livers of rats across 11 time points from fetus (-2 d), neonates (1, 7,

311   14 and 21 d), puberty (28 and 35 d), adulthood (60 and 180 d), to aging (540 and 800 d).

312   In general, there are three patterns of their expression: (1) The expressions of AhR,

313   Cyp1a1, Cyp2b1, Cyp2b2, Cyp3a1, Cyp3a2, and Ugt1a2 were low in fetal and neonatal

314   stage, increased with liver maturation and decreased extensively after 180 days; (2) the

315   majority of CYPs and other genes maintained relatively high levels throughout the

316   adulthood and decreased at aging of 540 and/or 800 days; (3) the expression of CAR

317   PXR, PPARα, Cyp4a1, Ugt1a2 had a first peak between 7-14 days of age. The protein

318   expression of CYP1A2, CYP2B1, CYP2E1, CYP3A1, CYP4A1, and CYP7A1 followed

319   the trend of mRNA changes. Characterization of CYPs in rat entire life span provides

320   fundamental information for drug metabolism and pharmacology studies in children and

321   elderly.

322   There are three unique features of the current study: (1) Most of the studies on age-

323   related changes in hepatic CYPs are performed in mice,(Cui et al. 2012; Hart et al. 2009;

324   Li et al. 2009) this study characterized CYPs and NRs in rats, another commonly-used

325   laboratory animals; (2) Most of the age-related changes in hepatic P450 are focused on

326   the developmental stages to maturation,(Asaoka et al. 2010; Bondarenko et al. 2016;

327   Cui et al. 2012; de Zwart et al. 2008; Kilanowicz et al. 2015; Park et al. 2016) and this

328   study covered the whole life span, and (3) this study extended our efforts to

329   characterize age- and sex-related changes in hepatic drug transporters(Hou et al. 2014;

330   Zhu et al. 2017) and defense mechanisms. (Hou WY 2014; Xu et al. 2018a; Xu et al.

331   2018b)

332

333   *P450-1 family*

334   CYP1 family is responsible for activation of toxicants and drugs. Cyp1a1 is

335   expressed very early in rodents and involved in developmental toxicity of

336   hexachloronaphthalene,(Kilanowicz et al. 2015) and immature rats has been proposed

337   as a potential model for chemical risks in children.(McPhail et al. 2016) Ontogeny of

338   hepatic CYP1A2 showed it rapidly increased after weanling, followed by significant

339   decrease during adulthood,(Elbarbry et al. 2007) However, CYP1A1/2 activity did not

340   change when fed Zuker diabetic fatty rats with high fat diet at 5 week (insulin resistant

341   stage) and 11-week (diabetic stage).(Park et al. 2016) Glycyrrhetinic acid potentiation

342   of clozapine hepatotoxicity is associated with CYP1A2 induction and suppression of

343   CYP2C11 and 2C13.(Jia et al. 2014)   CYP1A1 and CYP1A2 are regulated differently,

344   CYP1A1 decreased rapidly after maturation, while CYP1A2 remained the high

345   expression levels till 104 weeks.(Yun et al. 2010) The present results agreed with the

346   literature.

347

348   *P450-2 family*

349   Hepatic CYP2C2 and CYP2C11 decreased with age, along with CYP3A enzyme

350   genes.(Mori et al. 2007) In diethyl nitrosamine-induced liver insufficiency, the altered

351   expression of cytokines might contribute to CYP2C and CYP3A isoform regulation. (Kot

352   & Daujat-Chavanieu 2018) The expression of CYP2C11 is under the regulation of

353   growth hormones,(Das et al. 2014) as well as the dopaminergic

354 receptors.(Daskalopoulos et al. 2012) The expressions of CYP2C, CYP2E1, and

355 CYP3A are influenced by metabolic syndrome.(Bondarenko et al. 2016) CYP2C11 is a

356 male-specific CYP, its expression began to increase after weanling, and at puberty

357 reached 830-fold over the birth, and further dramatically increased over 100000-fold

358 over the birth level throughout the adulthood, but rapidly decreased at aging, consistent

359 with the literature.(Agrawal & Shapiro 2003; Das et al. 2014; Yun et al. 2010)

360 Age-associated CYP2E1 expression has important implications in pharmacology

361 and toxicology. Old rats have decreased CYP2E1, altered acetaminophen

362 pharmacokinetics, resulting in less sensitive to acetaminophen toxicity.(Mach et al. 2014)

363 On the other hand, higher CYP2E1 in young rats might be a reason of increased

364 sensitivity to isoniazid toxicity.(Mach et al. 2016) The observed expression pattern for

365 CYP2B1 (marked decreases at 800 days of age) and CYP2E1 (slight decreases at 800

366 days of age) are in agreement of the literature.(Yun et al. 2010) CYP2E1 plays roles in

367 thioacetamide hepatotoxicity, as Cyp2e1-/- mice are less sensitive to liver injury.(Kang

368 et al. 2008) Age-related differences in CYP2E1 levels can have important implications

369 for the toxic and carcinogenic actions of some hydrocarbons (e.g., benzene, hexane)

370 and short-chain halocarbons, such as carbon tetrachloride.(McPhail et al. 2016)

371

372

373 **P450-3 family**

374 CYP3 families are responsible for most drug metabolism.(Zanger & Schwab

375 2013) In rats with chronic kidney diseases, CYP3A and CYP2C mediated metabolism

376 are decreased.(Velenosi et al. 2012) Using liver microsomes (S9) from young and old

377 rats, severe metabolism impairment with aging for CYP3A and CYP2D substrates are

378 observed.(Salmin et al. 2017) CYP3A1 is higher in cells from young rats than in old

379 rats.(Czekaj et al. 2010) CYP3A in the liver are sensitive to aging with 50-70%

380 decreases, while CYP3A in the intestine is unchanged and in the kidney

381 increased.(Warrington et al. 2004) Generally speaking, hepatic CYP3A enzymes are

382 decreased with age.(Mori et al. 2007) In the present study, age-associated changes of

383 CYP3A1 and CYP3A2 followed "Pattern 1", while the expression of PXR, Cyp3a11

384 (mouse), and CYP3A4 protein followed "Pattern 2", a phenomenon in agreement of the

385 literature.(Mori et al. 2007; Yun et al. 2010)

386

3.4. P450-4 family

388   PPARα activation induces CYP4A1, together with acyl-CoA oxidase and SREBPs,

389 that play important roles in regulating lipid metabolism, especially in rats fed with high-

390 fat-diet.(Chang et al. 2011) The herbicide propaquizafop dose-dependently activates

391 PPARα and CYP4A, leading to increased liver weight and hypertrophy as a mode of

392 action in hepato-carcinogenesis.(Strupp et al. 2018) In addition to PPARα activation, the

393 induction of CYP4A by K^+PFOS also involves CAR and PXR activation,(Elcombe et al.

394 2012) leading to hepatomegaly. In the present study, the expression of PPARα,

395 CYP4A1, CAR, and PXR followed "Pattern 3". That is the first expression peak

396 appeared at 7-14 days of age, similar to that observed in mice.(Hart et al. 2009)

397

P450-7 family and BA homeostasis

399   Bile acid (BA) homeostasis is tightly regulated via a feedback loop operated by the

400 nuclear receptors FXR and small heterodimer partner (SHP). Loss of either FXR or SHP

401 alone, or Fxr-/-Shp-/- double knock out mice resulted in cholestasis and liver injury as

402 early as 3 weeks of age, and this dysfunction is linked to the dysregulation of bile acid

403 homeostatic key genes, particularly Cyp7a1.(Anakk et al. 2011) Hepatic CYP7A1 is a

404 rate-limiting enzyme that catabolizes cholesterol to bile acids, together with CYP8B1 as

405 the classic BA synthesis pathway, while CYP27A1 and CYP7B1 contribute to the

406 alternative pathway of BA biosynthesis.(Cuesta de Juan et al. 2007; Liu et al. 2014)

407 CYP27A1 is sensitive to inhibition by many xenobiotics.(Lam et al. 2018) In the present

408 study, the CYPs responsible for cholesterol metabolism and bile acid homeostasis

409 followed "Pattern 2", that was low at the neonatal stage, remained relative high levels

410 throughout the adulthood, and decreased at 800 days of age.

411   Taken together, CYPs are crucial enzymes in drug metabolism and

412 disposition.(Zanger & Schwab 2013) Induction or inhibition of CYPs have been

413 implicated in therapeutic efficacy and toxicity,(Jia et al. 2014; Mach et al. 2014; Mach et

414 al. 2016) and age-associated diseases.(Bondarenko et al. 2016; Velenosi et al. 2012)

415 especially in children(Kilanowicz et al. 2015; Li et al. 2017; McPhail et al. 2016) and in

416 elderly(McPhail et al. 2016; Salmin et al. 2017; Wauthier et al. 2007) Thus, a better

417 understanding of age-associated changes of CYPs is of significance for pharmacology,

418 toxicology, and therapeutics.

419

420 **UGT and SULT**

421   Glucuronidation and sulfation are two most important phase-II reactions to conjugate

422 the CYP-catalyzed metabolites for biliary or urinary elimination. Age has significant

423   impact on hepatic activities of glucuronidation and sulfation. For example, porcine

424   hepatic glucuronidation and sulfation activities were low at birth, peaked at 5-10 weeks,

425   and then declined at 20 weeks,(Hu 2017) similar to the observations in the present

426   study.

427   In childhood and adolescence, UGT expression can be affected by hormones and is

428   a reason of individual variation to medication.(Neumann et al. 2016) Compared to adults,

429   glucuronidation is reduced in children.(Krekels et al. 2012) UGT1A1 and UGT1A6 are

430   subjected to CAR and PPARα regulation,(Osabe et al. 2008) and age-associated

431   UGT1A1 changes are paralleled with CYP3A expression alterations in rats fed high-fat

432   diet.(Kawase et al. 2015; Osabe et al. 2008)

433   Sulfation is the most highly developed pathway during fetal development where

434   glucuronidation in particular is lacking.(Coughtrie 2015) Sulfation is normally a

435   detoxification reaction to facilitate the elimination of xenobiotics, although for some

436   molecules sulfation could be bioactivation.(Coughtrie 2016) The decreased Sult1a1

437   paralleled with major CYP metabolism genes in 600-day old rats,(Mori et al. 2007)

438   similar to current observations.

439

440   ***Conclusions***

441   Overall, the present study characterized age-related changes in a total of 25 CYP

442   isoforms and relevant genes in rat livers from development to aging. In general, these

443   genes are low in neonatal stages, increase with age, but decreased in aged animals,

444   and three expression patterns are characterized. These data could help our better

445   understanding of the effects of CYPs on drug metabolism, pharmacology, and

446   toxicology in the context of maturation and aging.

447

448   **Supplementary materials:** Supplemental Table 1 of qPCR primers can be found at

449   http://www.aspet/dmd.com

450   **Author Contributions:**

451   *Participate in research design:* Xu, Wu, Liu

452   *Conducted experiments:* Xu, Hu, Xie, Liu

453   *Performed data analysis:* Xu, Hu, Xie, Liu, Liu

454   *Wrote or contributed to the writing of the manuscript:* Xu, Hu, Xie, Wu, Liu

455   **Funding:** This study is supported by the National Natural Science Foundation of China

456   (81560592, 81560682).

457   **Conflicts of Interest:** The authors declare no conflict of interest.

458

459

460

461 **Figure Legends:**

462 **Figure 1. Age-related expression of CYP-1 family gene/proteins in livers of male**

463 **rats.** Livers from male SD rats at the fetus (-2 d before birth), the neonatal stage (1, 7,

464 and 14 d), and at weanling (21 d), at puberty (28 and 35 d), at adult (60 and 180 d), and

465 at aging (540 and 800 d), were collected to extract RNA, followed by qPCR analysis

466 (n=4-5 for each time point). *Significantly different from at birth, $p < 0.05$. For Western-

467 blot insert, the neonatal (14 d), at weanling (21 d), at puberty (28 d), at adult (60 and

468 180 d), and at aging (540 and 800 d) were collected to extract protein. Aliquoted

469 proteins (30 µg) were separated on NUPAGE 10% BT gels and the representative

470 western-blot was inserted into the figure (n=3). The molecular weight for CYP1A1 was

471 55 kD, and β-actin 43 kD.

472

473 **Figure 2. Age-related expression of CYP-2 family gene/proteins in livers of male**

474 **rats.** Livers from male SD rats at the fetus (-2 d before birth), the neonatal stage (1, 7,

475 and 14 d), and at weanling (21 d), at puberty (28 and 35 d), at adult (60 and 180 d), and

476 at aging (540 and 800 d), were collected to extract RNA, followed by qPCR analysis

477 (n=4-5 for each time point). *Significantly different from at birth, $p < 0.05$. For Western-

478 blot insert, the neonatal (14 d), at weanling (21 d), at puberty (28 d), at adult (60 and

479 180 d), and at aging (540 and 800 d) were collected to extract protein. Aliquoted

480 proteins (30 µg) were separated on NUPAGE 10% BT gels and the representative

481 western-blot was inserted into the figure (n=3). The molecular weight for CYP2B1 was

482 56 kD, CYP2E1 57 kD, and β-actin 43 kD.

483

484

485 **Figure 3. Age-related expression of CYP-3 family gene/proteins in livers of male**

486 **rats.** Livers from male SD rats at the fetus (-2 d before birth), the neonatal stage (1, 7,

487 and 14 d), and at weanling (21 d), at puberty (28 and 35 d), at adult (60 and 180 d), and

488 at aging (540 and 800 d), were collected to extract RNA, followed by qPCR analysis

489 (n=4-5 for each time point). *Significantly different from at birth, $p < 0.05$. For Western-

490 blot insert, the neonatal (14 d), at weanling (21 d), at puberty (28 d), at adult (60 and

491 180 d), and at aging (540 and 800 d) were collected to extract protein. Aliquoted

492 proteins (30 μg) were separated on NUPAGE 10% BT gels and the representative

493 western-blot was inserted into the figure (n=3). The molecular weight for CYP3A1 was

494 57 kD, and β-actin 43 kD.

495

496

497 **Figure 4. Age-related expression of CYP-4 family gene/proteins in livers of male**

498 **rats.** Livers from male SD rats at the fetus (-2 d before birth), the neonatal stage (1, 7,

499 and 14 d), and at weanling (21 d), at puberty (28 and 35 d), at adult (60 and 180 d), and

500 at aging (540 and 800 d), were collected to extract RNA, followed by qPCR analysis

501 (n=4-5 for each time point). For Western-blot insert, the neonatal (14 d), at weanling

502 (21 d), at puberty (28 d), at adult (60 and 180 d), and at aging (540 and 800 d) were

503 collected to extract protein. Aliquoted proteins (30 μg) were separated on NUPAGE 10%

504 BT gels and the representative western-blot was inserted into the figure (n=3). The

505 molecular weight for CYP4A1 was 59 kD, and β-actin 43 kD.

506

507

Figure 5. Age-related expression of CYP-7 family gene/proteins in livers of male

rats. Livers from male SD rats at the fetus (-2 d before birth), the neonatal stage (1, 7,

and 14 d), and at weanling (21 d), at puberty (28 and 35 d), at adult (60 and 180 d),

and at aging (540 and 800 d), were collected to extract RNA, followed by qPCR analysis

(n=4-5 for each time point). *Significantly different from at birth, $p < 0.05$. For Western-

blot insert, the neonatal (14 d), at weanling (21 d), at puberty (28 d), at adult (60 and

180 d), and at aging (540 and 800 d) were collected to extract protein. Aliquoted

proteins (30 μg) were separated on NUPAGE 10% BT gels and the representative

western-blot was inserted into the figure (n=3). The molecular weight for CYP7A1 was

55 kD, and β-actin 43 kD.

518

519

Figure 6. Age-related mRNA expression of UGT and SULT family genes in livers

of male rats. Livers from male SD rats at the fetus (-2 d before birth), the neonatal

stage (1, 7, and 14 d), and at weanling (21 d), at puberty (28 and 35 d), at adult (60 and

180 d), and at aging (540 and 800 d), were collected to extract RNA, followed by real-

time RT-PCR analysis (n=4-5 for each time point). *Significantly different from at birth,

$p < 0.05$.

526

527

References

Agrawal AK, and Shapiro BH. 2003. Constitutive and inducible hepatic cytochrome P450 isoforms in senescent male and female rats and response to low-dose phenobarbital. *Drug Metab Dispos* 31:612-619.

Aleksunes LM, and Klaassen CD. 2012. Coordinated regulation of hepatic phase I and II drug-metabolizing genes and transporters using AhR-, CAR-, PXR-, PPARalpha-, and Nrf2-null mice. *Drug Metab Dispos* 40:1366-1379. 10.1124/dmd.112.045112

Anakk S, Watanabe M, Ochsner SA, McKenna NJ, Finegold MJ, and Moore DD. 2011. Combined deletion of Fxr and Shp in mice induces Cyp17a1 and results in juvenile onset cholestasis. *J Clin Invest* 121:86-95. 10.1172/jci42846

Asaoka Y, Sakai H, Sasaki J, Goryo M, Yanai T, Masegi T, and Okada K. 2010. Changes in the gene expression and enzyme activity of hepatic cytochrome P450 in juvenile Sprague-Dawley rats. *J Vet Med Sci* 72:471-479.

Bondarenko LB, Shayakhmetova GM, Voronina AK, and Kovalenko VM. 2016. Age-dependent features of CYP3A, CYP2C, and CYP2E1 functioning at metabolic syndrome. *J Basic Clin Physiol Pharmacol* 27:603-610. 10.1515/jbcpp-2016-0012

Chang CJ, Tzeng TF, Liou SS, Chang YS, and Liu IM. 2011. Kaempferol regulates the lipid-profile in high-fat diet-fed rats through an increase in hepatic PPARalpha levels. *Planta Med* 77:1876-1882. 10.1055/s-0031-1279992

Coughtrie MW. 2015. Ontogeny of Human Conjugating Enzymes. *Drug Metab Lett* 9:99-108.

Coughtrie MWH. 2016. Function and organization of the human cytosolic sulfotransferase (SULT) family. *Chem Biol Interact* 259:2-7. 10.1016/j.cbi.2016.05.005

Cuesta de Juan S, Monte MJ, Macias RI, Wauthier V, Calderon PB, and Marin JJ. 2007. Ontogenic development-associated changes in the expression of genes involved in rat bile acid homeostasis. *J Lipid Res* 48:1362-1370. 10.1194/jlr.M700034-JLR200

Cui JY, Renaud HJ, and Klaassen CD. 2012. Ontogeny of novel cytochrome P450 gene isoforms during postnatal liver maturation in mice. *Drug Metab Dispos* 40:1226-1237. 10.1124/dmd.111.042697

Czekaj P, Bryzek A, Czekaj TM, Koryciak-Komarska H, Wiaderkiewicz A, Plewka D, and Sieron AL. 2010. Cytochrome P450 mRNA expressions along with in vitro differentiation of hepatocyte precursor cells from fetal, young and old rats. *Folia Histochem Cytobiol* 48:46-57. 10.2478/v10042-008-0085-5

Das RK, Banerjee S, and Shapiro BH. 2014. Irreversible perinatal imprinting of adult expression of the principal sex-dependent drug-metabolizing enzyme CYP2C11. *Faseb j* 28:4111-4122. 10.1096/fj.13-248864

Daskalopoulos EP, Lang MA, Marselos M, Malliou F, and Konstandi M. 2012. D(2)-dopaminergic receptor-linked pathways: critical regulators of CYP3A, CYP2C, and CYP2D. *Mol Pharmacol* 82:668-678. 10.1124/mol.112.078709

de Zwart L, Scholten M, Monbaliu JG, Annaert PP, Van Houdt JM, Van den Wyngaert I, De Schaepdrijver LM, Bailey GP, Coogan TP, Coussement WC, and Mannens GS. 2008. The ontogeny of drug metabolizing enzymes and transporters in the rat. *Reprod Toxicol* 26:220-230. 10.1016/j.reprotox.2008.09.010

Durnas C, Loi CM, and Cusack BJ. 1990. Hepatic drug metabolism and aging. *Clin Pharmacokinet* 19:359-389. 10.2165/00003088-199019050-00002

Elbarbry FA, McNamara PJ, and Alcorn J. 2007. Ontogeny of hepatic CYP1A2 and CYP2E1 expression in rat. *J Biochem Mol Toxicol* 21:41-50. 10.1002/jbt.20156

574   Elcombe CR, Elcombe BM, Foster JR, Chang SC, Ehresman DJ, and Butenhoff JL. 2012. Hepatocellular
575   hypertrophy and cell proliferation in Sprague-Dawley rats from dietary exposure to potassium
576   perfluorooctanesulfonate results from increased expression of xenosensor nuclear receptors
577   PPARalpha and CAR/PXR. *Toxicology* 293:16-29. 10.1016/j.tox.2011.12.014
578   Hart SN, Cui Y, Klaassen CD, and Zhong XB. 2009. Three patterns of cytochrome P450 gene expression
579   during liver maturation in mice. *Drug Metab Dispos* 37:116-121. 10.1124/dmd.108.023812
580   He XJ, Ejiri N, Nakayama H, and Doi K. 2005. Effects of pregnancy on CYPs protein expression in rat liver.
581   *Exp Mol Pathol* 78:64-70. 10.1016/j.yexmp.2004.08.011
582   Hines RN. 2008. The ontogeny of drug metabolism enzymes and implications for adverse drug events.
583   *Pharmacol Ther* 118:250-267. 10.1016/j.pharmthera.2008.02.005
584   Hines RN. 2013. Developmental expression of drug metabolizing enzymes: impact on disposition in
585   neonates and young children. *Int J Pharm* 452:3-7. 10.1016/j.ijpharm.2012.05.079
586   Hou WY, Xu SF, Zhu QN, Lu YF, Cheng XG, and Liu J. 2014. Age- and sex-related differences of organic
587   anion-transporting polypeptide gene expression in livers of rats. *Toxicol Appl Pharmacol*
588   280:370-377. 10.1016/j.taap.2014.08.020
589   Hou WY ZD, Zhu QN, Xu SF, Wu Q, Lu YF, Liu J. 2014. Age-dependent expression of metallothionein in
590   livers of rats *Journal of Zunyi Medical College* 37:111-116.
591   Hu SX. 2017. Age-related change of hepatic uridine diphosphate glucuronosyltransferase and
592   sulfotransferase activities in male chickens and pigs. *J Vet Pharmacol Ther* 40:270-278.
593   10.1111/jvp.12355
594   Jia LL, Zhong ZY, Li F, Ling ZL, Chen Y, Zhao WM, Li Y, Jiang SW, Xu P, Yang Y, Hu MY, Liu L, and Liu XD.
595   2014. Aggravation of clozapine-induced hepatotoxicity by glycyrrhetinic acid in rats. *J Pharmacol*
596   *Sci* 124:468-479.
597   Kang JS, Wanibuchi H, Morimura K, Wongpoomchai R, Chusiri Y, Gonzalez FJ, and Fukushima S. 2008.
598   Role of CYP2E1 in thioacetamide-induced mouse hepatotoxicity. *Toxicol Appl Pharmacol*
599   228:295-300. 10.1016/j.taap.2007.11.010
600   Kawase A, Ito A, Yamada A, and Iwaki M. 2015. Age-related changes in mRNA levels of hepatic
601   transporters, cytochrome P450 and UDP-glucuronosyltransferase in female rats. *Eur J Drug*
602   *Metab Pharmacokinet* 40:239-244. 10.1007/s13318-014-0208-7
603   Kilanowicz A, Czekaj P, Sapota A, Skrzypinska-Gawrysiak M, Bruchajzer E, Darago A, Czech E, Plewka D,
604   Wiaderkiewicz A, and Sitarek K. 2015. Developmental toxicity of hexachloronaphthalene in
605   Wistar rats. A role of CYP1A1 expression. *Reprod Toxicol* 58:93-103.
606   10.1016/j.reprotox.2015.09.005
607   Kirby GM, Batist G, Alpert L, Lamoureux E, Cameron RG, and Alaoui-Jamali MA. 1996. Overexpression of
608   cytochrome P-450 isoforms involved in aflatoxin B1 bioactivation in human liver with cirrhosis
609   and hepatitis. *Toxicol Pathol* 24:458-467. 10.1177/019262339602400408
610   Kot M, and Daujat-Chavanieu M. 2018. Altered cytokine profile under control of the serotonergic system
611   determines the regulation of CYP2C11 and CYP3A isoforms. *Food Chem Toxicol* 116:369-378.
612   10.1016/j.fct.2018.04.051
613   Krekels EH, Danhof M, Tibboel D, and Knibbe CA. 2012. Ontogeny of hepatic glucuronidation; methods
614   and results. *Curr Drug Metab* 13:728-743.
615   Lam M, Mast N, and Pikuleva IA. 2018. Drugs and Scaffold That Inhibit Cytochrome P450 27A1 In Vitro
616   and In Vivo. *Mol Pharmacol* 93:101-108. 10.1124/mol.117.110742
617   Li H, Canet MJ, Clarke JD, Billheimer D, Xanthakos SA, Lavine JE, Erickson RP, and Cherrington NJ. 2017.
618   Pediatric Cytochrome P450 Activity Alterations in Nonalcoholic Steatohepatitis. *Drug Metab*
619   *Dispos* 45:1317-1325. 10.1124/dmd.117.077644

620   Li Y, Cui Y, Hart SN, Klaassen CD, and Zhong XB. 2009. Dynamic patterns of histone methylation are
621   associated with ontogenic expression of the Cyp3a genes during mouse liver maturation. *Mol*
622   *Pharmacol* 75:1171-1179. 10.1124/mol.108.052993
623   Liu J, Lu H, Lu YF, Lei X, Cui JY, Ellis E, Strom SC, and Klaassen CD. 2014. Potency of individual bile acids to
624   regulate bile acid synthesis and transport genes in primary human hepatocyte cultures. *Toxicol*
625   *Sci* 141:538-546. 10.1093/toxsci/kfu151
626   Mach J, Huizer-Pajkos A, Cogger VC, McKenzie C, Le Couteur DG, Jones BE, de Cabo R, and Hilmer SN.
627   2014. The effect of aging on acetaminophen pharmacokinetics, toxicity and Nrf2 in Fischer 344
628   rats. *J Gerontol A Biol Sci Med Sci* 69:387-397. 10.1093/gerona/glt095
629   Mach J, Huizer-Pajkos A, Mitchell SJ, McKenzie C, Phillips L, Kane A, Jones B, de Cabo R, Cogger V, Le
630   Couteur DG, and Hilmer SN. 2016. The effect of ageing on isoniazid pharmacokinetics and
631   hepatotoxicity in Fischer 344 rats. *Fundam Clin Pharmacol* 30:23-34. 10.1111/fcp.12157
632   Matalova P, Urbanek K, and Anzenbacher P. 2016. Specific features of pharmacokinetics in children.
633   *Drug Metab Rev* 48:70-79. 10.3109/03602532.2015.1135941
634   McPhail BT, White CA, Cummings BS, Muralidhara S, Wilson JT, and Bruckner JV. 2016. The immature rat
635   as a potential model for chemical risks to children: Ontogeny of selected hepatic P450s. *Chem*
636   *Biol Interact* 256:167-177. 10.1016/j.cbi.2016.07.005
637   Mori K, Blackshear PE, Lobenhofer EK, Parker JS, Orzech DP, Roycroft JH, Walker KL, Johnson KA, Marsh
638   TA, Irwin RD, and Boorman GA. 2007. Hepatic transcript levels for genes coding for enzymes
639   associated with xenobiotic metabolism are altered with age. *Toxicol Pathol* 35:242-251.
640   10.1080/01926230601156286
641   Neumann E, Mehboob H, Ramirez J, Mirkov S, Zhang M, and Liu W. 2016. Age-Dependent Hepatic UDP-
642   Glucuronosyltransferase Gene Expression and Activity in Children. *Front Pharmacol* 7:437.
643   10.3389/fphar.2016.00437
644   Osabe M, Sugatani J, Fukuyama T, Ikushiro S, Ikari A, and Miwa M. 2008. Expression of hepatic UDP-
645   glucuronosyltransferase 1A1 and 1A6 correlated with increased expression of the nuclear
646   constitutive androstane receptor and peroxisome proliferator-activated receptor alpha in male
647   rats fed a high-fat and high-sucrose diet. *Drug Metab Dispos* 36:294-302.
648   10.1124/dmd.107.017731
649   Park SY, Kim CH, Lee JY, Jeon JS, Kim MJ, Chae SH, Kim HC, Oh SJ, and Kim SK. 2016. Hepatic expression
650   of cytochrome P450 in Zucker diabetic fatty rats. *Food Chem Toxicol* 96:244-253.
651   10.1016/j.fct.2016.08.010
652   Saghir SA, Khan SA, and McCoy AT. 2012. Ontogeny of mammalian metabolizing enzymes in humans and
653   animals used in toxicological studies. *Crit Rev Toxicol* 42:323-357.
654   10.3109/10408444.2012.674100
655   Salmin SF, Giroux MC, Vachon P, and Beaudry F. 2017. In vitro metabolism of specific CYP2D and CYP3A
656   opioid substrates using rat liver S9 fractions and mass spectrometry reveal a severe metabolic
657   impairment with increasing age. *Biomed Chromatogr* 31. 10.1002/bmc.3786
658   Shi S, and Klotz U. 2011. Age-related changes in pharmacokinetics. *Curr Drug Metab* 12:601-610.
659   Strupp C, Bomann WH, Spezia F, Gervais F, Forster R, Richert L, and Singh P. 2018. A human relevance
660   investigation of PPARalpha-mediated key events in the hepatocarcinogenic mode of action of
661   propaquizafop in rats. *Regul Toxicol Pharmacol* 95:348-361. 10.1016/j.yrtph.2018.04.005
662   Velenosi TJ, Fu AY, Luo S, Wang H, and Urquhart BL. 2012. Down-regulation of hepatic CYP3A and CYP2C
663   mediated metabolism in rats with moderate chronic kidney disease. *Drug Metab Dispos*
664   40:1508-1514. 10.1124/dmd.112.045245
665   Wang H, Li W, Muhammad I, Sun X, Cui X, Cheng P, Qayum A, and Zhang X. 2018. Biochemical basis for
666   the age-related sensitivity of broilers to aflatoxin B1. *Toxicol Mech Methods* 28:361-368.
667   10.1080/15376516.2018.1428258

668   Warrington JS, Greenblatt DJ, and von Moltke LL. 2004. Age-related differences in CYP3A expression and
669   activity in the rat liver, intestine, and kidney. *J Pharmacol Exp Ther* 309:720-729.
670   10.1124/jpet.103.061077
671   Wauthier V, Verbeeck RK, and Calderon PB. 2007. The effect of ageing on cytochrome p450 enzymes:
672   consequences for drug biotransformation in the elderly. *Curr Med Chem* 14:745-757.
673   Xu S, Hou D, Liu J, and Ji L. 2018a. Age-associated changes in GSH S-transferase gene/proteins in livers of
674   rats. *Redox Rep* 23:213-218. 10.1080/13510002.2018.1546985
675   Xu SF, Ji LL, Wu Q, Li J, and Liu J. 2018b. Ontogeny and aging of Nrf2 pathway genes in livers of rats. *Life*
676   *Sci* 203:99-104. 10.1016/j.lfs.2018.04.018
677   Yun KU, Oh SJ, Oh JM, Kang KW, Myung CS, Song GY, Kim BH, and Kim SK. 2010. Age-related changes in
678   hepatic expression and activity of cytochrome P450 in male rats. *Arch Toxicol* 84:939-946.
679   10.1007/s00204-010-0520-1
680   Zanger UM, and Schwab M. 2013. Cytochrome P450 enzymes in drug metabolism: regulation of gene
681   expression, enzyme activities, and impact of genetic variation. *Pharmacol Ther* 138:103-141.
682   10.1016/j.pharmthera.2012.12.007
683   Zhu QN, Hou WY, Xu SF, Lu YF, and Liu J. 2017. Ontogeny, aging, and gender-related changes in hepatic
684   multidrug resistant protein genes in rats. *Life Sci* 170:108-114. 10.1016/j.lfs.2016.11.022
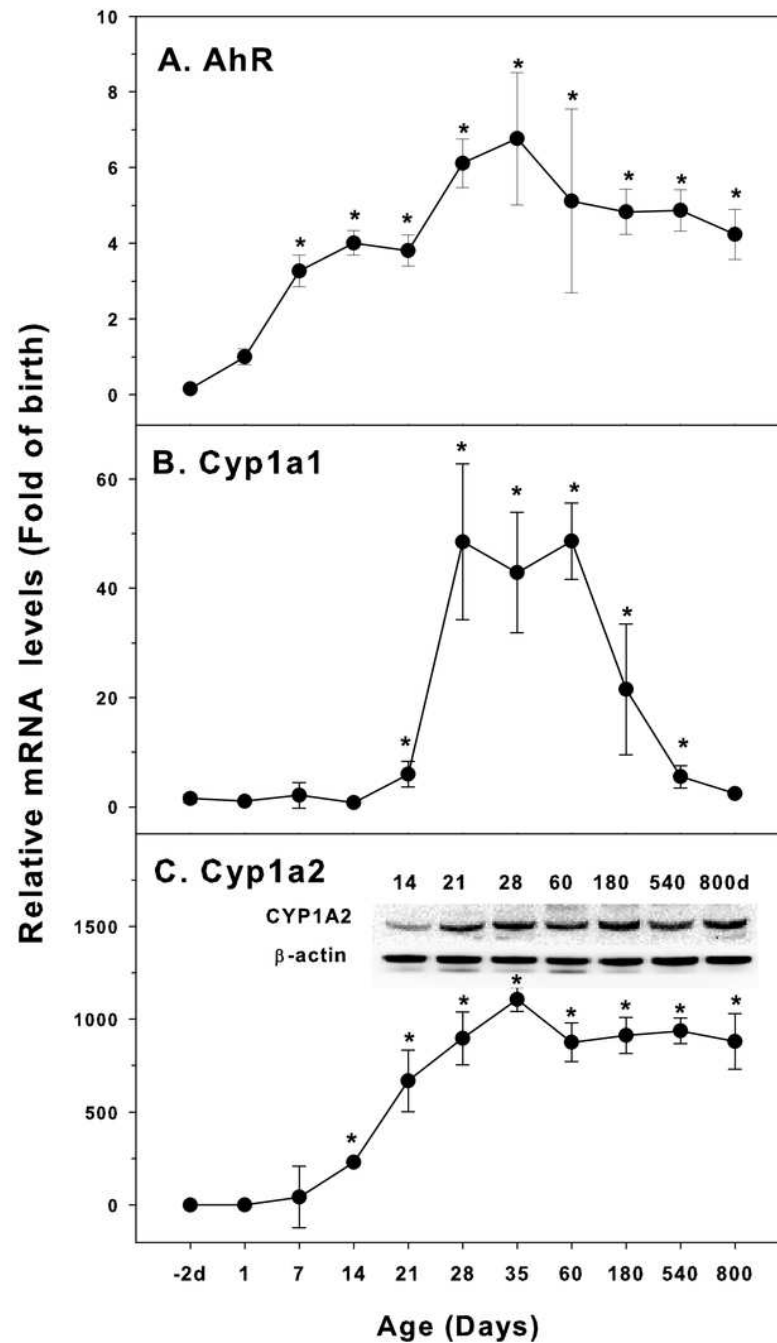
685

Figure 1

Figure 1. Age-related expression of CYP-1 family gene/proteins in livers of male rats.

Livers from male SD rats at the fetus (-2 d before birth), the neonatal stage (1, 7, and 14 d), and at weanling (21 d), at puberty (28 and 35 d), at adult (60 and 180 d), and at aging (540 and 800 d), were collected to extract RNA, followed by qPCR analysis (n=4-5 for each time point). *Significantly different from at birth, $p < 0.05$. For Western-blot insert, the neonatal (14 d), at weanling (21 d), at puberty (28 d), at adult (60 and 180 d), and at aging (540 and 800 d) were collected to extract protein. Aliquoted proteins (30 μg) were separated on NUPAGE 10% BT gels and the representative western-blot was inserted into the figure (n=3). The molecular weight for CYP1A1 was 55 kD, and β-actin 43 kD.

Figure 2

Figure 2. Age-related expression of CYP-2 family gene/proteins in livers of male rats.

Livers from male SD rats at the fetus (-2 d before birth), the neonatal stage (1, 7, and 14 d), and at weanling (21 d), at puberty (28 and 35 d), at adult (60 and 180 d), and at aging (540 and 800 d), were collected to extract RNA, followed by qPCR analysis (n=4-5 for each time point). *Significantly different from at birth, $p < 0.05$. For Western-blot insert, the neonatal (14 d), at weanling (21 d), at puberty (28 d), at adult (60 and 180 d), and at aging (540 and 800 d) were collected to extract protein. Aliquoted proteins (30 μg) were separated on NUPAGE 10% BT gels and the representative western-blot was inserted into the figure (n=3). The molecular weight for CYP2B1 was 56 kD, CYP2E1 57 kD, and β-actin 43 kD.
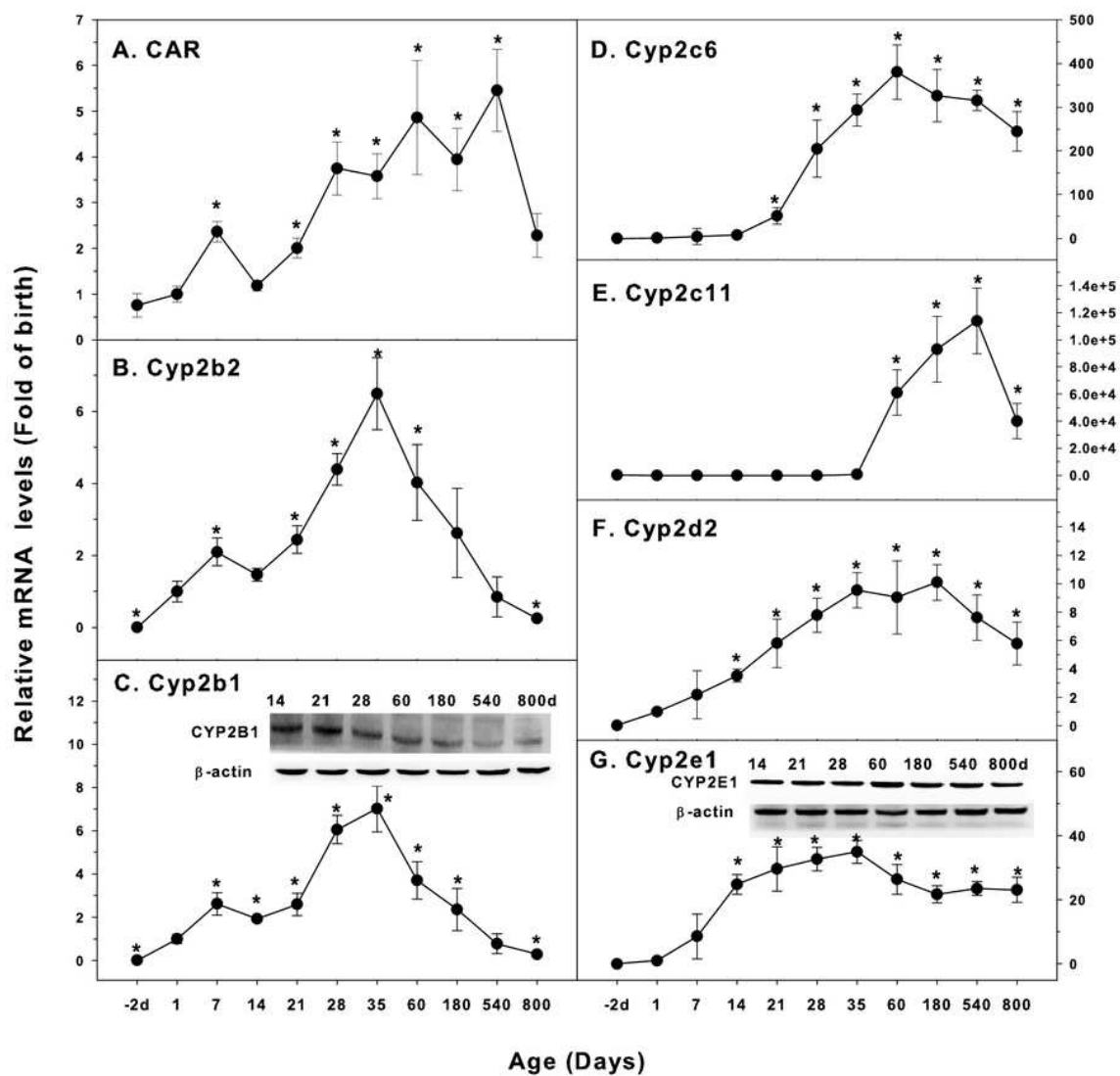
Figure 3

Figure 3. Age-related expression of CYP-3 family gene/proteins in livers of male rats.

Livers from male SD rats at the fetus (-2 d before birth), the neonatal stage (1, 7, and 14 d), and at weanling (21 d), at puberty (28 and 35 d), at adult (60 and 180 d), and at aging (540 and 800 d), were collected to extract RNA, followed by qPCR analysis (n=4-5 for each time point). *Significantly different from at birth, $p < 0.05$. For Western-blot insert, the neonatal (14 d), at weanling (21 d), at puberty (28 d), at adult (60 and 180 d), and at aging (540 and 800 d) were collected to extract protein. Aliquoted proteins (30 µg) were separated on NUPAGE 10% BT gels and the representative western-blot was inserted into the figure (n=3). The molecular weight for CYP3A1 was 57 kD, and β-actin 43 kD.
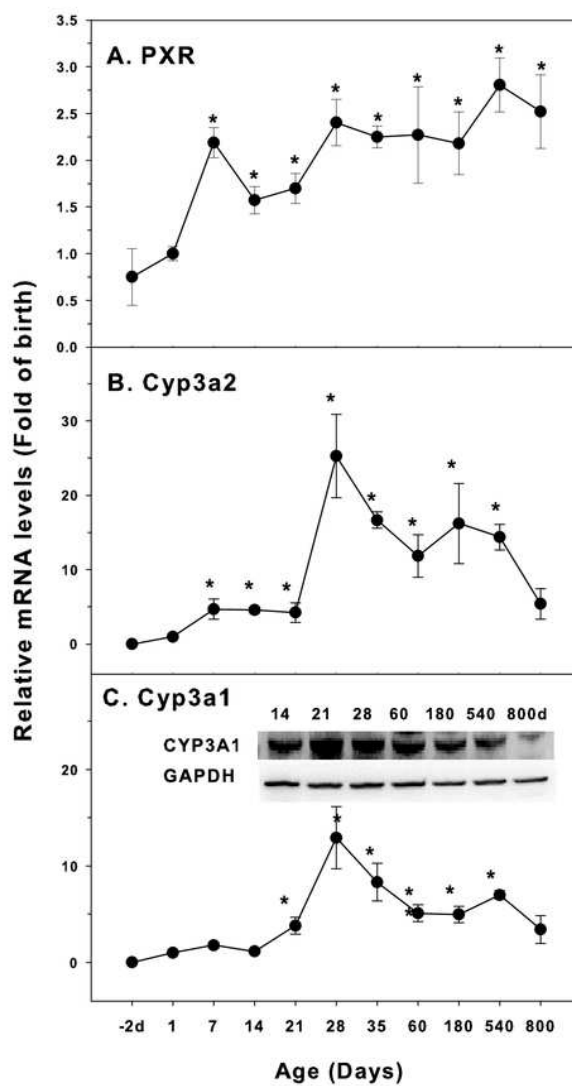
Figure 4

Figure 4. Age-related expression of CYP-4 family gene/proteins in livers of male rats.

Livers from male SD rats at the fetus (-2 d before birth), the neonatal stage (1, 7, and 14 d), and at weanling (21 d), at puberty (28 and 35 d), at adult (60 and 180 d), and at aging (540 and 800 d), were collected to extract RNA, followed by qPCR analysis (n=4-5 for each time point). For Western-blot insert, the neonatal (14 d), at weanling (21 d), at puberty (28 d), at adult (60 and 180 d), and at aging (540 and 800 d) were collected to extract protein. Aliquoted proteins (30 µg) were separated on NUPAGE 10% BT gels and the representative western-blot was inserted into the figure (n=3). The molecular weight for CYP4A1 was 59 kD, and β-actin 43 kD.
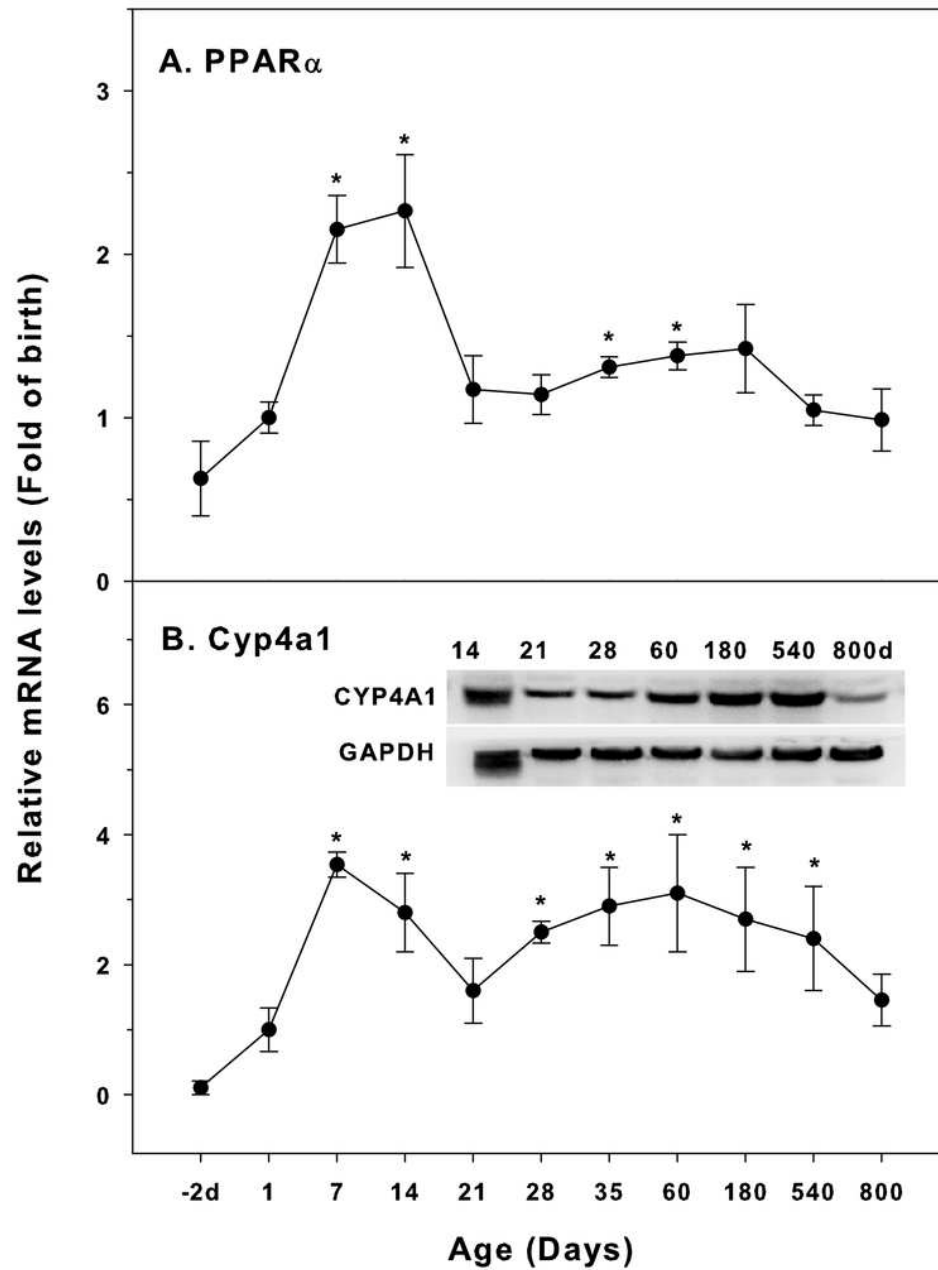
Figure 5

Figure 5. Age-related expression of CYP-7 family gene/proteins in livers of male rats.

Livers from male SD rats at the fetus (-2 d before birth), the neonatal stage (1, 7, and 14 d), and at weanling (21 d), at puberty (28 and 35 d), at adult (60 and 180 d), and at aging (540 and 800 d), were collected to extract RNA, followed by qPCR analysis (n=4-5 for each time point). *Significantly different from at birth, $p < 0.05$. For Western-blot insert, the neonatal (14 d), at weanling (21 d), at puberty (28 d), at adult (60 and 180 d), and at aging (540 and 800 d) were collected to extract protein. Aliquoted proteins (30 µg) were separated on NUPAGE 10% BT gels and the representative western-blot was inserted into the figure (n=3). The molecular weight for CYP7A1 was 55 kD, and β-actin 43 kD.
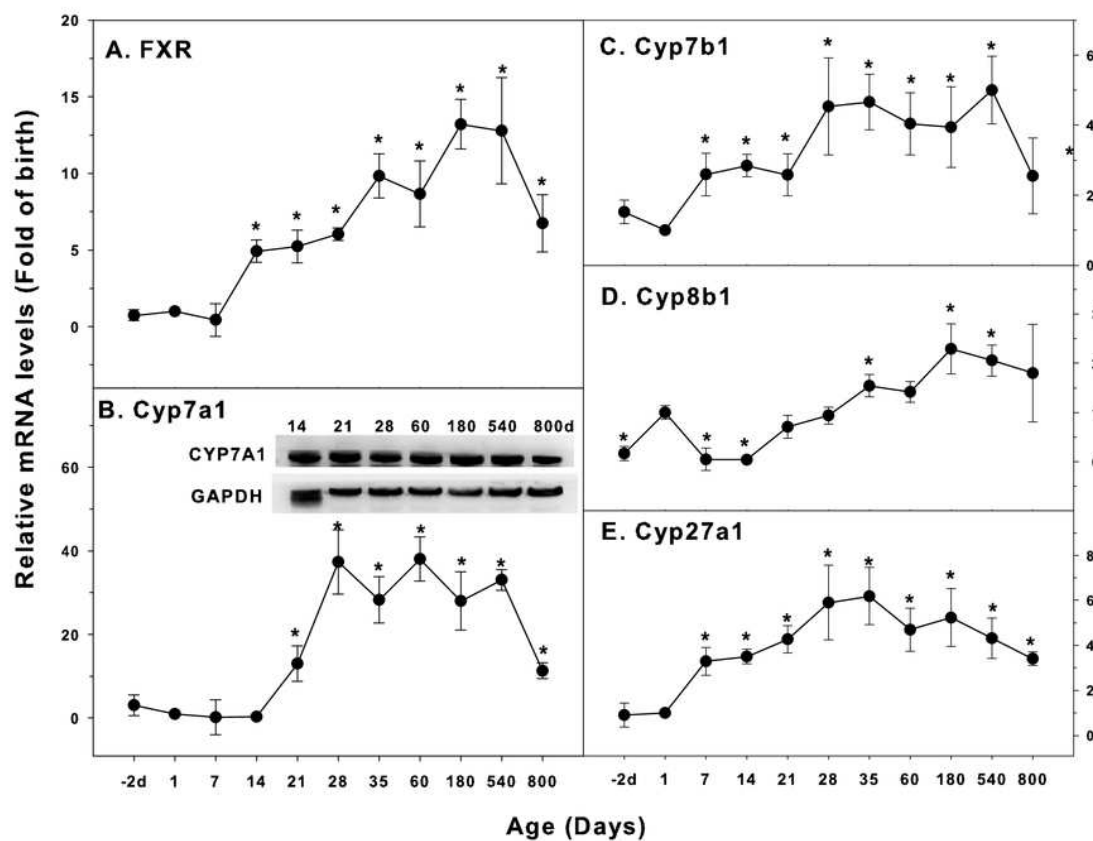
Figure 6

Figure 6. Age-related mRNA expression of UGT and SULT family genes in livers of male rats.

Livers from male SD rats at the fetus (-2 d before birth), the neonatal stage (1, 7, and 14 d), and at weanling (21 d), at puberty (28 and 35 d), at adult (60 and 180 d), and at aging (540 and 800 d), were collected to extract RNA, followed by real-time RT-PCR analysis (n=4-5 for each time point). *Significantly different from at birth, $p < 0.05$.