DMD Fast Forward. Published on February 22, 2017 as DOI: 10.1124/dmd.116.074583
This article has not been copyedited and formatted. The final version may differ from this version.

Determination of Human Hepatic CYP2C8 and CYP1A2 Age-Dependent Expression to Support Human Health Risk Assessment for Early Ages

Gina Song, Xueying Sun, Ronald N. Hines, D. Gail McCarver, Brian G. Lake, Thomas G. Osimitz, Moire R. Creek, Harvey J. Clewell and Miyoung Yoon

The Hamner Institutes for Health Sciences, RTP, NC, 27709, USA (GS, XS, HJC and MY)

ScitoVation, LLC, RTP, NC, USA (GS, XS, HJC and MY)

U.S. Environmental Protection Agency, RTP, NC, 27711, USA (RNH)

Medical College of Wisconsin, Milwaukee, WI, 53226, USA (DGM)

Centre for Toxicology, University of Surrey, Surrey, GU2 7XH, UK (BGL)

Science Strategies, LLC, Charlottesville, VA, 22902, USA (TGO)

Valent USA Corporation, Walnut Creek, CA, 94596, USA (MRC)

Running Title: The Ontogeny of CYP2C8 and CYP1A2 in Humans

Text Pages: 31

Number of Tables: 1

Number of Figures: 8

Number of References: 42

Words in Abstract (<250 words): 232

Words in Introduction (<750 words): 700

Words in Discussion (<1500 words): 1,498

Non-standard Abbreviations:

CYP: Cytochrome P450

CES: Carboxylesterase

IVIVE: in vitro to in vivo extrapolation

PCA: Post-conceptual age

Correspondence Author:

Miyoung Yoon, Ph.D.

Senior Principal Investigator

ScitoVation, LLC.

6 Davis Drive

P.O. Box 110566

Research Triangle Park, NC 27709, USA

Tel: +1 919 558-1340

Email: myoon@scitovation.com

Abstract

Predicting age-specific metabolism is important for evaluating age-related drug and chemical sensitivity. Multiple cytochrome P450s (CYP) and carboxylesterase (CES) enzymes are responsible for human pyrethroid metabolism. Complete ontogeny data for each enzyme is needed to support *in vitro* to *in vivo* extrapolation (IVIVE). This study was designed to determine age-dependent human hepatic CYP2C8 expression, for which only limited ontogeny data are available, and to further define CYP1A2 ontogeny. CYP2C8 and 1A2 protein levels were measured by quantitative Western blotting using liver microsomal samples prepared from 222 subjects with ages ranging from 8 weeks gestation to 18 years after birth. The median CYP2C8 expression was significantly greater among samples from subjects older than 35 postnatal days (n=122) compared to fetal samples and those from very young infants (fetal to 35 days postnatal, n=100) (0.00 vs. 13.38 pmol/mg microsomal protein; $p<0.0001$). In contrast, the median CYP1A2 expression was significantly greater after 15 months postnatal age (n=55) than in fetal and younger postnatal samples (fetal to 15 months postnatal, n=167) (0.0167 vs. 2.354 pmol/mg microsomal protein; $p<0.0001$). CYP2C8, but not CYP1A2, protein levels, significantly correlated with those of CYP2C9, CYP2C19, and CYP3A4 ($p<0.001$) consistent with CYP2C8 and CYP1A2 ontogeny being probably controlled by different mechanisms. This study provides key data for physiologically based pharmacokinetic model-based prediction of age-dependent pyrethroid metabolism, which will be used for IVIVE to support pyrethroid risk assessment for early life stages.

DMD Fast Forward. Published on February 22, 2017 as DOI: 10.1124/dmd.116.074583
This article has not been copyedited and formatted. The final version may differ from this version.

INTRODUCTION

Substantial changes occur in physiological and biochemical processes during development and growth (Clewell et al., 2002). Such changes can substantially affect chemical disposition in the growing body resulting in age-related differences in therapeutic efficacy or toxicity (Hines, 2008; Miyagi et al., 2012). Developmental changes in the expression of xenobiotic metabolizing enzyme systems must be taken into account to better understand age-dependent sensitivity to chemicals as metabolism can be a major determinant of compound disposition and/or toxicity. The increased synthetic pyrethroid deltamethrin neurotoxicity and lethality observed in young rats is a good example of age-related sensitivity due to immature metabolizing enzyme systems overwhelmed by the high chemical doses typically applied in rodent toxicity studies (Sheets et al., 1994; Anand et al., 2006). Juvenile susceptibility to pyrethroids in animal studies led to the present concern over potential differential sensitivity of pyrethroid insecticides between juveniles and adults due to their predominant exposure to humans in residential settings (Heudorf and Angerer, 2001; Schettgen et al., 2002).

Hepatic cytochrome P450 (CYPs) and carboxylesterases (CES) are important enzymes involved in pyrethroid metabolism and clearance in both rats and humans (Anand et al., 2006; Godin et al., 2006; Ross et al., 2006; Crow et al., 2007; Godin et al., 2007; Scollon et al., 2009). The CYP enzymes are capable of catalyzing oxidative transformation of the pyrethroids (Scollon et al., 2009) and the CES enzymes exhibit hydrolysis of some, but not all pyrethroids in the human (Ross et al., 2006). It is noted that significant species differences exist in the relative importance of various CYP and CES enzymes responsible for pyrethroid metabolism and detoxification. In the rat, the oxidative reactions of the pyrethroids are dominated by CYP1A1, 1A2, 2C6, 2C11, and 3A1, whereas CYP2C8, 2C19, and 3A4 are the predominant contributors in the human (Godin et

DMD Fast Forward. Published on February 22, 2017 as DOI: 10.1124/dmd.116.074583
This article has not been copyedited and formatted. The final version may differ from this version.

al., 2007; Scollon et al., 2009). In contrast to the rat, human CES1 is important for the hydrolysis of several pyrethroids (Ross et al., 2006). In addition, serum esterases demonstrate significant activity in the rat, but not in the human (Crow et al., 2007). Moreover, metabolic competency for some pyrethroids develops late in rats as indicated by age-dependent increase in hepatic intrinsic clearance of deltamethrin in rats (Anand et al., 2006; Kim et al., 2010). However, because of clear species differences in the contribution of each CYP and/or CES enzyme to metabolism of a given pyrethroid, it is difficult to generalize age-dependency in metabolism in humans from rodent data. Thus, information on the developmental changes of the human CYP and CES enzymes involved in pyrethroid metabolisms are critical to examine the juvenile sensitivity in humans.

The developmental trajectories of human CYP2C8, CYP2C19, CYP3A4, CES1, and CES2, the major metabolic contributors to pyrethroid metabolisms, have been well characterized (Stevens et al., 2003; Koukouritaki et al., 2004; Hines, 2008; Scollon et al., 2009; Hines, 2012; Hines et al., 2016). However, limited knowledge is available on the ontogeny of human CYP2C8 despite its potential importance in pyrethroid metabolism (Scollon et al., 2009) and its abundance in the human liver (Yeo et al., 2004; Achour et al., 2014). Johnson *et al*. (2006) demonstrated that the development of CYP2C8 with age was best described by a hyperbolic model, but they had incomplete data on early ages (0-2 years only). Naraharisetti *et al*. (2010) characterized hepatic CYP2C8 protein expression in individuals with different CYP2C8 genotypes, but their study failed to include early life ages (median age: 46 years, range: 7-70 years). In addition to CYP2C8, human CYP1A2 is expected to demonstrate pyrethroid metabolic activity and the available CYP1A2 data is insufficient for *in vitro* to *in vivo* extrapolation (IVIVE) (Scollon et al., 2009).

The objective of this study was to determine age-dependent expression levels of human hepatic CYP2C8 and CYP1A2 to fill the current data gap of enzyme ontogeny information, using

a human liver tissue bank (Koukouritaki et al., 2004). A total of 222 human liver microsome samples were examined, with ages ranging from 8 weeks gestation to 18 years after birth. Our findings will support IVIVE modelling to predict pyrethroid metabolism for different ages by incorporating enzyme ontogeny and expressed enzyme kinetic data. Similar approaches can be applied to predict age-appropriate metabolism parameters for other environmental compounds.

MATERIALS AND METHODS

Human liver tissue bank. The microsomal samples consisting of 222 cryopreserved microsomal liver tissue fractions obtained from donors ranging in age from 8 weeks gestation to 18 years after birth were obtained from the Brain and Tissue Bank for Developmental Disorders, University of Maryland Baltimore and University of Miami (National Institute of Child Health and Human Development contract HD-83284) and the Central Laboratory for Human Embryology at the University of Washington (National Institute of Child Health and Human Development contract HD-00836). Liver microsomal fractions were prepared by differential centrifugation as described in Koukouritaki *et al*. (2004) and stored at 80˚C until used for Western blotting analysis. The same samples have been used previously to characterize the developmental expression pattern of other microsomal and cytosolic proteins. Samples from individuals with disease processes that potentially would have involved liver pathology were excluded and more information on clinical exclusion criteria is described in the Supplemental Methods. The demographics of the donors are presented in **Table 1**. The median age was 56 days postnatal age with 61% of male and 34% female donors. The collection and described use of these tissue samples was considered exempt by the Children's Hospital and Health System of Wisconsin Institutional Review Board.

Other materials. Primary polyclonal antibody raised in rabbit against human CYP2C8 was obtained from GeneTex (Irvine, CA) (catalogue number GTX113666, lot number 40464). Primary monoclonal antibody raised in mouse against human CYP1A2 was obtained from GeneTex (Irvine, CA) (catalogue number GTX84639, lot number 821403197). Purified secondary antibody against CYP2C8 (catalogue number 926-68021, lot number C40415-04) and purified secondary antibody against CYP1A2 (catalogue number 926-32210, lot number C40528-02) were obtained from LI-COR Biosciences (Lincoln, NE). Specificity of the primary antibodies was verified by

evaluating cross-reactivity of the CYP2C8 and 1A2 primary antibodies against the same loading amounts of other cytochrome P450s including CYP1A1, 3A4, 3A5, 1B1, 2B6, 2C9, 2C18, 2C19, 2D6, 2E1, and 2J2 and CYP1A1, 2A6, 3A4, 3A5, 1B1, 2B6, 2C8, 2C9, 2C18, 2C19, 2D6, and 2E1, respectively. Purified recombinant human CYP2C8 protein (catalogue number 456252) and human CYP1A2 protein (catalogue number 456203) were obtained from Corning (Woburn, MA). The other CYP proteins which were used for cross-reactivity evaluation were also obtained from Corning (Woburn, MA). XTreme 200 pooled human liver microsomes (catalogue number H2620) were obtained from Xenotech (Kansas City, KS).

Protein molecular weight markers were obtained from Life Technologies (catalogue number LC5602; Carlsbad, CA) and dual color standards were obtained from Bio-Rad (catalogue number 161-0374; Richmond, CA). Novex® 4-12% Tris-Glycine mini gels were purchased from Life Technologies (catalogue number EC60385BOX; Carlsbad, CA). SuperSignal Western Blot enhancer was purchased from Thermo Scientific (catalogue number 46640; Rockford, IL). Immobilon-P-PVDF transfer membrane was purchased from Millipore Corporation (catalogue number IPVH07850; Billerica, MA).

Quantitative Western blot analysis. CYP2C8 and 1A2 expressions in human liver microsomal samples (n=222) were immunochemically measured by Western blot analysis as described previously (Naraharisetti et al., 2010). Preliminary studies were performed to determine CYP2C8 and 1A2 expression across all ages. Based on the preliminary results, the individual hepatic microsomal samples, either 5 or 10 µg, were separated by SDS-polyacrylamide gel electrophoresis (PAGE) along with purified recombinant CYP2C8 and 1A2 as standards (0.005-1 pmole of CYP2C8, and 0.0002-0.04 pmole of CYP1A2) and then transferred onto PVDF membranes. Each membrane was incubated with 5 mL of antigen pretreatment solution at room temperature for 10

DMD Fast Forward. Published on February 22, 2017 as DOI: 10.1124/dmd.116.074583
This article has not been copyedited and formatted. The final version may differ from this version.

minutes with shaking, and subsequently incubated with a mixture of anti-CYP2C8 (1:500 dilution) or anti-CYP1A2 (1:500 dilution) antibody containing primary antibody diluent at 4°C overnight. Subsequently, each membrane was rinsed with Tris-buffered saline (TBS) containing 0.1% Tween-20, and then incubated with the corresponding secondary antibodies (1:5000 dilution) for 1 hour at room temperature. Blots were scanned using an Odyssey Infrared Imaging Systems (LI-COR Biosciences, Lincoln, NE). In each gel, besides unknown samples and one set of standard samples, a 2 µg of commercial pooled human liver microsome (XenoTech, Kansas City, KS) was added as a quality control sample for interday variation.

The interday coefficients of variation for the microsomal CYP2C8 and CYP1A2 (n=19) were 11.3% and 9.2%, respectively. Non-linear regression analysis was used to construct a calibration curve to quantify the amount of both CYP2C8 and CYP1A2 protein in each individual sample based on the integrated band intensity observed with the respective purified, recombinant proteins. A one site binding regression equation, Y=Bmax*X/(Kd+X), provided in GraphPad Prism (version 6.0, GraphPad Software, Inc., La Jolla, CA) was used, where X is the loading amount of each recombinant protein in pmoles and Y is the band density integrated using Image Studio™ Lite software (version 4.0, LI-COR Biosciences, Lincoln, NE). An $r^2 \geq 0.99$ was accepted as evidence of good fit for the standard curve derived from known amounts of either CYP2C8 (0.005 – 1 pmole) or 1A2 (0.0002 -0.04 pmole) (Supplemental Table S1).

Statistical analysis. Scatter plots of CYP2C8-and CYP1A2-specific content as a function of age were used to examine overall trends. Classification and regression tree (CART) analysis (R, Version 3.1.1) was used to evaluate differences in CYP2C8- and CYP1A2-specific content among different age groups. Kruskal-Wallis nonparametric tests were used for statistical comparisons between age groups followed by adjustment for multiple comparisons using Dunn's post hoc test

(GraphPad Prism version 7.01, La Jolla, CA). Other variables were also evaluated using nonparametric testing (GraphPad Prism version 7.01, La Jolla, CA). Linear regression was used to examine relationships between continuous variables (R version 3.1.1) and the strength of the r^2 was considered as highly relevant (*i.e.*, proximity to 1) in addition to the *p* value. Stepwise regression testing (multivariate analysis) was used to evaluate factors potentially contributing to variation in enzyme protein expression (R version 3.1.1). *P* value of less than 0.05 was considered statistically significant. All statistical tests were two-sided.

DMD Fast Forward. Published on February 22, 2017 as DOI: 10.1124/dmd.116.074583
This article has not been copyedited and formatted. The final version may differ from this version.

RESULTS

Western Blot Analysis of Human Hepatic CYP2C8 and CYP1A2

Western blot analyses revealed a major immunoreactive band at 56kDa corresponding to CYP2C8, whereas for CYP1A2, a single, major immunoreactive band was detected at 58kDa. Representative Western blots are shown in **Figure 1A** and **1B**. The upper band in parallel with CYP2C8 shown in tested human liver microsomal samples (shown in **Figure 1A**) was suspected to be CYP2C9 due to relative cross-reactivity of the CYP2C8 polyclonal antibody with CYP2C9 (**Supplemental Figure S1**). However, this signal did not affect the quantitation of CYP2C8 because CYP2C8 was electrophoretically well separated from the suspected CYP2C9 band under the current experimental conditions. Anti-CYP1A2 antibody was specific to CYP1A2 and did not cross-react with any other recombinant P450 proteins tested in the current study (**Supplemental Figure S2**). No cross-reactivity between the anti-CYP2C8 antibody and CYP1A2 protein or between the anti-CYP1A2 antibody and CYP2C8 protein was observed (data not shown).

Ontogeny and Inter-individual Variation in CYP2C8

Human CYP2C8 was detectable in the majority of postnatal samples (142 out of 163 samples). With outliers, defined as having specific contents outside 1.5 times the 25^{th} to 75^{th} precentiles, included, the median of microsomal CYP2C8 was 12.62 pmoles/mg microsomal protein (minimum: 0.148 and maximum: 77.98 pmoles/mg microsomal protein); however, only considering the 5^{th} to 95^{th} percentile range, the overall distribution of CYP2C8 content for the entire sample varied about 80-fold. The fetal and early infancy developmental expression patterns for CYP2C8 are presented **in Figure 2A** and **B**, respectively. With the exception of three outliers, CYP2C8 was either not expressed or expressed at very low levels (<5 pmoles/mg microsomal protein) during the human fetal period (n=60). However, CYP2C8 was readily detected in most

DMD Fast Forward. Published on February 22, 2017 as DOI: 10.1124/dmd.116.074583
This article has not been copyedited and formatted. The final version may differ from this version.

postnatal with expression occurring as early as the first day of life in some, but not all, microsomal samples (**Figure 2B**). No subjects older than 91 days had non-detectable CYP2C8 expression (**Figure 2B**).

Classification tree analysis and confirmatory Kruskal Wallis testing of all prenatal and postnatal samples revealed that microsomal CYP2C8 content was significantly lower among the prenatal samples combined with samples from young infants (<35 days of age) compared to the older age group (**Figure 3A**). This age differential appeared to be driven by the onset of CYP2C8 expression after birth and progressive increase at 35 days of age (**Figure 3A**). CYP2C8 protein levels in the age range of 8 weeks gestation to 35 days postnatal age were less than 1 % of those in the age range of 11-18 years. A second node at 11 years was identified by the initial tree analysis but it was not statistically significant (p=0.15) when considered in conjunction with the 35 day cut point (**Figure 3A** and **B)**. Thus, samples from fetuses and individuals less than 35 days old postnatally (N=100, median (Interquartile range, IQR) = 0.00 (0.00-0.87) pmoles/mg microsomal protein) were significantly lower than those from postnatal individuals ages between 35 days and 11 years (N=104, median (IQR) = 12.76 (7.48-22.72) pmoles/mg microsomal protein) ($p<0.0001$; Kruskal-Wallis test, **Figure 4**), however, the microsomal CYP2C8 expression among individuals in the later age group was not statistically different from that in the greater than 11 years of age group (N=18, median (IQR) = 20.33 (14.78-39.83) pmoles/mg microsomal protein; **Figure 4**).

Ontogeny and Inter-individual Variation in CYP1A2

Human CYP1A2 was also immunodetectable in the majority of postnatal samples (101 out of 163). With outliers as defined previously included, the median of microsomal CYP1A2 was 0.316 pmoles/mg microsomal protein (minimum: 0.00467 and maximum: 15.54 pmoles/mg microsomal protein), whereas CYP1A2 content ranged from 0.00772 to 5.497 pmoles/mg

DMD Fast Forward. Published on February 22, 2017 as DOI: 10.1124/dmd.116.074583
This article has not been copyedited and formatted. The final version may differ from this version.

microsomal protein without considering outliers. Similar to human CYP2C8, CYP1A2 expression was essentially absent during the gestational period (**Figure 5A**). After birth, CYP1A2 expression levels were not readily observed in the majority of samples as seen in fetal samples, indicating that birth may not impact CYP1A2 expression content. In addition, high interindividual variability was observed among samples from postnatal individuals ages 0 to 1 years (**Figure 5B**).

Classification tree analysis and confirmatory Kruskal Wallis testing revealed that microsomal CYP1A2 content was significantly lower in fetuses and individuals less than 15 months of age (N=167, median (IQR) = 0.017 (0.00-0.206) pmoles/mg microsomal protein) compared to older age groups (N=55, median (IQR) = 2.354 (0.815-4.587) pmoles/mg microsomal protein) (p<0.0001; Mann Whitney test, **Figure 6 and Figure 7**). This age differential appears attributable to the delayed expression onset in the young, as well as increased CYP1A2 expression among older individuals.

Factors Affecting Developmental Expression of CYP2C8 and CYP1A2

Human CYP2C8-specific content was present at significantly higher levels than CYP1A2-specific content among all samples during the gestational period and postnatal development (paired t test, p<0.0001). There was a positive correlation between values of CYP2C8 and CYP1A2 protein content among both the pre- and postnatal age groups (linear regression, r^2=0.55 and r^2=0.34, respectively; p<0.0001, each comparison), consistent with the findings from the recent meta-analysis study (Achour et al., 2014). Both CYP2C8 and CYP1A2 contents in pre- and postnatal samples were related to age (stepwise linear regression, r^2=0.15 and r^2=0.26, respectively; p<0.0001). Gender was not related to either CYP2C8- (median (IQR) pmoles/mg microsomal protein, male (N=136): 5.73 (0.00-18.30), female (N=76): 6.51 (0.00-18.38), Mann Whitney U, p>0.05) or CYP1A2-specific content (median (IQR) pmoles/mg microsomal protein,

DMD Fast Forward. Published on February 22, 2017 as DOI: 10.1124/dmd.116.074583
This article has not been copyedited and formatted. The final version may differ from this version.

male: 0.13 (0.00-1.02), female: 0.09 (0.01-1.52), Mann Whitney *U*, $p>0.05$). When age was considered simultaneously, the influence of gender on either CYP2C8 or CYP1A2 enzyme content continued to be non-significant ($p>0.05$). Of note, with age considered simultaneously, race/ethnicity group was no longer significantly associated with CYP2C8 or CYP1A2 expression. There was no correlation between the postmortem interval (time between death and freezing of liver samples) and the CYP1A2 expression levels (linear regression, $r^2=0.017$; $p>0.05$), whereas there was a positive, although minor relationship between the postmortem interval and the amount of CYP2C8 (linear regression, $r^2=0.049$; $p<0.01$). Age was still significantly associated with both CYP2C8 and 1A2 protein expression levels after adjustment for postmortem interval. With all factors considered simultaneously, including age, gender, race/ethnic group, and postmortem interval, only age remained significantly associated with human hepatic microsomal CYP2C8 and CYP1A2 content (stepwise linear regression, $p<0.001$).

Correlations between CYP2C Family and CYP3C4 Protein Content

Considering some reported shared regulatory mechanisms between human cytochrome P450 enzymes (Honkakoski and Negishi, 2000), CYP2C8 protein levels were compared to the previously reported data for CYP2C9, 2C19, and CYP3A4 protein content determined with similar methods using the same tissue samples (Stevens et al., 2003; Koukouritaki et al., 2004). In contrast to CYP2C8, microsomal CYP2C9 and CYP2C19 are readily detected in fetal and early life hepatic samples (**Figure 8**). Overall, microsomal CYP2C8 protein expression levels were modestly correlated with those of CYP2C9 (linear regression, $r^2=0.193$, $p<0.001$), CYP2C19 ($r^2=0.321$, $p<0.001$), and CYP3A4 ($r^2=0.304$, $p<0.001$), suggesting that CYP2C8 may share some developmental regulatory mechanisms with these other cytochromes P450s.

DISCUSSION

Age-dependent maturation of xenobiotic metabolizing enzymes can profoundly impact chemical kinetics in the developing body. In the past several years, the growing knowledge of enzyme ontogeny has contributed greatly to improving our qualitative and quantitative understanding of age-related differences in chemical sensitivity or drug efficacy/adversity. Such evaluation is possible when enzyme ontogeny data is used in combination with computational modeling tools such as physiologically based pharmacokinetics (PBPK) modeling, which provides an estimate of the target tissue concentration under given exposure conditions based on *in vitro*-based information (Clewell et al., 2004; Jiang et al., 2013). A major challenge in early life PBPK modeling, however, is the difficulty in obtaining extensive biochemical, especially metabolic data, for pediatric populations. To overcome this challenge, a bottom-up process called "*in vitro* to *in vivo* extrapolation (IVIVE) has been increasingly used to support parameterization of the PBPK models (Clewell et al., 2004; Johnson et al., 2006; Rostami-Hodjegan and Tucker, 2007; Yoon et al., 2012). Among the available choices of *in vitro* assay systems for age-appropriate metabolism parameters, human recombinant enzymes have several advantages compared to cell-based or tissue-derived materials. Fewer sample quality uncertainties are expected in the estimated *in vivo* metabolic constants derived by combining recombinant enzyme activity with enzyme content data compared to those extrapolated from the data obtained from subcellular fractions or primary hepatocytes collected from age-specific tissue samples. In addition, human metabolic variability can be addressed based on observed variation in enzyme abundance instead of using a large number of individual donors or volunteers to assess activity. A substantial amount of data have been collected resulting in a database describing the ontogeny of the majority of the key xenobiotic

metabolizing enzymes in humans; an extensive review of these data can be found in Hines (2008 and 2012).

CYP2C8 and CYP1A2 are major hepatic cytochromes P450, comprising about 7% and 4-16% of total microsomal cytochrome P450 content in the adult human liver (Zanger and Schwab, 2013). Both enzymes play an important role in carrying out oxidative metabolisms of several therapeutic drugs and environmental chemicals including pyrethroids (Godin et al., 2007; Guengerich, 2008; Scollon et al., 2009). However, there is inadequate knowledge on human CYP2C8 and CYP1A2 ontogeny. Previously reported studies were limited in sample size at early ages (Cresteil et al., 1982; Lee et al., 1991; Johnson et al., 2006; Naraharisetti et al., 2010). In this study, for the first time, human hepatic CYP2C8 and CYP1A2 was determined using a large number of prenatal and postnatal human liver tissue samples.

Both CYP2C8 and CYP1A2 expression were essentially absent during the gestational period, consistent with increased postnatal expression of both enzymes being linked to birth. However, the developmental expression patterns after birth were different. Significant CYP2C8 expression was observed in early infant samples with an approximate 8-fold difference in CYP2C8 levels between individuals less than 35 days postnatal age and older donors (>35 days to 18 years). In contrast, CYP1A2 protein expression exhibited no significant change between the fetal and neonatal periods (~30 days). A progressive increase (~10 fold) in CYP1A2-specific contents was observed after 15 months postnatal age, indicating a delayed CYP1A2 ontogenesis consistent with earlier findings based on more limited data sets (Sonnier and Cresteil, 1998). Early quantification studies using probe substrates and polyclonal antibodies demonstrated CYP1A2 was either not expressed or was expressed at very low levels in human fetal tissues (Cresteil et al., 1982; Lee et al., 1991). The study by Sonnier and Cresteil (1998) supported a delayed CYP1A2 ontogeny by

reporting that CYP1A2 was absent in fetal and neonatal microsomal livers while samples from one to three month old infants (n=23) attained 10-15% of the adult values and samples from three months to 1 year old infants reached 20 to 25% of adult values. Thus, our findings on CYP1A2 are consistent with earlier reports.

After the specific age thresholds for significant increases in CYP2C8- and CYP1A2-specific content, microsomal CYP2C8 and CYP1A2 protein expression did not exhibit additional significant age-related differences. This suggests that if any developmental changes in CYP2C8 and CYP1A2 activity occur after about postnatal 35 days and 15 months, respectively, such changes would be modest and may depend on exogenous factors (Hines, 2008; Hines, 2012). Naraharisetti *et al*. (2010) explored human liver CYP2C8 expression and effects of genotype (*CYP2C8*3* or **4*), gender, and age on mRNA and/or protein expression levels. Inter-individual variation in CYP2C8 mRNA and protein expression in 60 liver tissues from Caucasian donors was not shown to be affected by genetic variation, age, or gender. Given that their studies were enriched with adult samples (median: 46 years, range: 7-70 years), the lack of an observable age effect on CYP2C8 protein expression levels is consistent with our findings. Thus, our results augment the existing, but limited knowledge of human CYP2C8 and 1A2 ontogeny and will enable more accurate assessment of developmental changes in the disposition, efficacy and toxicity of drugs and environmental chemicals.

The variation observed in the overall distribution of CYP2C8 and CYP1A2 content as well as the variation within individual age groups was substantial. Several studies have identified CYP2C8 as highly polymorphic among various ethnic populations, the most common alleles being *CYP2C8*2* and *CYP2C8*3* (Totah and Rettie, 2005). *CYP2C8*2* is expressed most commonly in African Americans with an allele frequency of 18%, but is very rare in Caucasians and Japanese

DMD Fast Forward. Published on February 22, 2017 as DOI: 10.1124/dmd.116.074583
This article has not been copyedited and formatted. The final version may differ from this version.

(Bahadur et al., 2002). In contrast, *CYP2C8*3* is most commonly expressed in Caucasians with an allele frequency of 23% but is quite rare in African Americans and almost absent in Japanese (Soyama et al., 2001; Soyama et al., 2002). Both *CYP2C8*2* and **3* alleles are reported to result in changes in amino acid sequence, leading to altered enzyme expression levels and metabolic activity toward therapeutic drugs (*e.g.*, paclitaxel) and endogenous substrate (*e.g.*, arachidonic acid) *in vitro* and *in vivo* (Soyama et al., 2001; Bahadur et al., 2002; Soyama et al., 2002). In contrast, the clinical relevance of multiple CYP1A2 polymorphisms (*e.g.*, *CYP1A2*1* or **6*) is controversial (Jiang et al., 2006). Thus, the presence of CYP2C8 genetic polymorphisms among the liver tissue donors may contribute to the considerable variability observed in CYP2C8-specific content within and between age groups. Diet influences normal cytochrome P450-dependent metabolic maturation processes. For example, using caffeine and dextromethorphan as probes, respectively, formula-fed infants displayed increased CYP1A1 and CYP3A4 metabolic activity compared to breast-fed infants (Le Guennec and Billon, 1987; Blake et al., 2006). CYP2C8 and CYP1A2 protein expression and metabolic activity also have been shown to be induced by therapeutic drugs and smoking (Totah and Rettie, 2005; Zanger and Schwab, 2013). These observations implicate genetic polymorphisms and postnatal exposure to dietary and/or other inducing/suppressing agents (*e.g.*, rifampin and carbamazepine) as having a significant role in the developmental regulation of cytochrome P450 expression and functional activity from early infancy (Blake et al., 2006) and likely explains at least some of the interindividual differences in CYP2C8 and 1A2 expression observed in the current study.

Constitutive human CYP1A2 expression only occurs in liver (Zanger and Schwab, 2013). CYP1A2 expression also is regulated by the aryl hydrocarbon receptor (AhR) and by other receptors (Pelkonen and Hakkola, 2008), including the constitutive androstane receptor (AhR) and

pregnane X receptor (PXR). Numerous xenobiotics serve as AhR, CAR amd PXR agonistic ligands and thus, can induce *CYP1A2* transcription (Blake et al., 2006; Pelkonen and Hakkola, 2008; Zanger and Schwab, 2013). In contrast, human CYP2C8 is characterized by extrahepatic expression (Klose et al., 1999) as well as its abundance in the liver (Zanger and Schwab, 2013) and is considered the most inducible member of the human CYP2C subfamily (Totah and Rettie, 2005). Three nuclear receptors, PXR, CAR, and the glucocorticoid receptor, are known to be involved in *CYP2C8* induction (Gerbal-Chaloin et al., 2001; Ferguson et al., 2005). These same nuclear receptors are also involved in the induction and regulation of other *CYP2C* family genes (*CYP2C9* and *CYP2C19*) and *CYP3A4* (Gerbal-Chaloin et al., 2001; Ferguson et al., 2005). Thus, the correlation of hepatic CYP2C8 with CYP2C9, 2C19, and 3A4 protein levels in the same individuals may be attributed to shared factors that regulate hepatic-specific expression during development (Koukouritaki et al., 2004).

Establishment of an *in vitro* and in silico-based evaluation strategy in conjunction with relevant human exposure information is of great importance to risk and safety assessment of potentially vulnerable populations and life-stages. Our data will fill current data gaps in enzyme ontogeny information for IVIVE-supported prediction of age-dependent drug and chemical metabolisms. As an example, IVIVE calculation and *in vivo* metabolic clearance for a hypothetical compound X, for which CYP1A2 is a major enzyme involved in hepatic metabolism, are described in Supplemental Table S2 and Figure S3, respectively. The immediate utility of the data from the current study is to predict age-appropriate metabolism parameters for pyrethroid PBPK models. However, the value of the ontogeny data for these two enzymes is broader, providing the ability to accurately predict age-dependent hepatic clearance for a variety of chemicals, thereby increasing our confidence in risk and safety decisions for early human life stages.

ACKNOWLEDGEMENTS

The authors certify that their freedom to design, conduct, interpret, and publish research was not compromised by any sponsor.

AUTHORSHIP CONTRIBUTIONS

Participated in research design: Hines, McCarver, Osimitz, Creek, Clewell, and Yoon

Conducted experiments: Sun

Contributed new reagents or analytic tools: Hines

Performed data analysis: Song, McCarver and Yoon

Wrote or contributed to the writing of the manuscript: Song, Sun, Hines, McCarver, Lake, Osimitz, Creek, and Yoon

REFERENCES

Achour B, Barber J, and Rostami-Hodjegan A (2014) Expression of hepatic drug-metabolizing cytochrome p450 enzymes and their intercorrelations: a meta-analysis. *Drug Metab Dispos* **42:**1349-1356.

Anand SS, Kim KB, Padilla S, Muralidhara S, Kim HJ, Fisher JW, and Bruckner JV (2006) Ontogeny of hepatic and plasma metabolism of deltamethrin in vitro: role in age-dependent acute neurotoxicity. *Drug Metab Dispos* **34:**389-397.

Bahadur N, Leathart JB, Mutch E, Steimel-Crespi D, Dunn SA, Gilissen R, Houdt JV, Hendrickx J, Mannens G, Bohets H, Williams FM, Armstrong M, Crespi CL, and Daly AK (2002) CYP2C8 polymorphisms in Caucasians and their relationship with paclitaxel 6alpha-hydroxylase activity in human liver microsomes. *Biochemical pharmacology* **64:**1579-1589.

Blake MJ, Abdel-Rahman SM, Pearce RE, Leeder JS, and Kearns GL (2006) Effect of diet on the development of drug metabolism by cytochrome P-450 enzymes in healthy infants. *Pediatric research* **60:**717-723.

Clewell HJ, Gentry PR, Covington TR, Sarangapani R, and Teeguarden JG (2004) Evaluation of the potential impact of age- and gender-specific pharmacokinetic differences on tissue dosimetry. *Toxicological sciences : an official journal of the Society of Toxicology* **79:**381-393.

Clewell HJ, Teeguarden J, McDonald T, Sarangapani R, Lawrence G, Covington T, Gentry R, and Shipp A (2002) Review and evaluation of the potential impact of age- and gender-specific pharmacokinetic differences on tissue dosimetry. *Critical reviews in toxicology* **32:**329-389.

Cresteil T, Beaune P, Kremers P, Flinois JP, and Leroux JP (1982) Drug-metabolizing enzymes in human foetal liver: partial resolution of multiple cytochromes P 450. *Pediatric pharmacology (New York, NY)* **2:**199-207.

Crow JA, Borazjani A, Potter PM, and Ross MK (2007) Hydrolysis of pyrethroids by human and rat tissues: examination of intestinal, liver and serum carboxylesterases. *Toxicology and applied pharmacology* **221:**1-12.

Ferguson SS, Chen Y, LeCluyse EL, Negishi M, and Goldstein JA (2005) Human CYP2C8 is transcriptionally regulated by the nuclear receptors constitutive androstane receptor, pregnane X receptor, glucocorticoid receptor, and hepatic nuclear factor 4alpha. *Molecular pharmacology* **68:**747-757.

Gerbal-Chaloin S, Pascussi JM, Pichard-Garcia L, Daujat M, Waechter F, Fabre JM, Carrere N, and Maurel P (2001) Induction of CYP2C genes in human hepatocytes in primary culture. *Drug Metab Dispos* **29:**242-251.

Godin SJ, Crow JA, Scollon EJ, Hughes MF, DeVito MJ, and Ross MK (2007) Identification of rat and human cytochrome p450 isoforms and a rat serum esterase that metabolize the pyrethroid insecticides deltamethrin and esfenvalerate. *Drug Metab Dispos* **35:**1664-1671.

Godin SJ, Scollon EJ, Hughes MF, Potter PM, DeVito MJ, and Ross MK (2006) Species differences in the in vitro metabolism of deltamethrin and esfenvalerate: differential oxidative and hydrolytic metabolism by humans and rats. *Drug Metab Dispos* **34:**1764-1771.

Guengerich FP (2008) Cytochrome p450 and chemical toxicology. *Chemical research in toxicology* **21:**70-83.

Heudorf U and Angerer J (2001) Metabolites of pyrethroid insecticides in urine specimens: current exposure in an urban population in Germany. *Environ Health Perspect* **109:**213-217.

Hines RN (2008) The ontogeny of drug metabolism enzymes and implications for adverse drug events. *Pharmacology & therapeutics* **118:**250-267.

Hines RN (2012) Age-Dependent Expression of Human Drug-Metabolizing Enzymes. *Encyclopedia of Drug Metabolism and Interactions*.

Hines RN, Simpson PM, and McCarver DG (2016) Age-Dependent Human Hepatic Carboxylesterase 1 (CES1) and Carboxylesterase 2 (CES2) Postnatal Ontogeny. *Drug Metab Dispos* **44:**959-966.

Honkakoski P and Negishi M (2000) Regulation of cytochrome P450 (CYP) genes by nuclear receptors. *The Biochemical journal* **347:**321-337.

Jiang XL, Zhao P, Barrett JS, Lesko LJ, and Schmidt S (2013) Application of physiologically based pharmacokinetic modeling to predict acetaminophen metabolism and pharmacokinetics in children. *CPT: pharmacometrics & systems pharmacology* **2:**e80.

Jiang Z, Dragin N, Jorge-Nebert LF, Martin MV, Guengerich FP, Aklillu E, Ingelman-Sundberg M, Hammons GJ, Lyn-Cook BD, Kadlubar FF, Saldana SN, Sorter M, Vinks AA, Nassr N, von Richter O, Jin L, and Nebert DW (2006) Search for an association between the human CYP1A2 genotype and CYP1A2 metabolic phenotype. *Pharmacogenetics and genomics* **16:**359-367.

Johnson TN, Rostami-Hodjegan A, and Tucker GT (2006) Prediction of the clearance of eleven drugs and associated variability in neonates, infants and children. *Clinical pharmacokinetics* **45:**931-956.

Kim KB, Anand SS, Kim HJ, White CA, Fisher JW, Tornero-Velez R, and Bruckner JV (2010) Age, dose, and time-dependency of plasma and tissue distribution of deltamethrin in immature rats. *Toxicological sciences : an official journal of the Society of Toxicology* **115:**354-368.

Klose TS, Blaisdell JA, and Goldstein JA (1999) Gene structure of CYP2C8 and extrahepatic distribution of the human CYP2Cs. *Journal of biochemical and molecular toxicology* **13:**289-295.

Koukouritaki SB, Manro JR, Marsh SA, Stevens JC, Rettie AE, McCarver DG, and Hines RN (2004) Developmental expression of human hepatic CYP2C9 and CYP2C19. *The Journal of pharmacology and experimental therapeutics* **308:**965-974.

Le Guennec JC and Billon B (1987) Delay in caffeine elimination in breast-fed infants. *Pediatrics* **79:**264-268.

Lee QH, Fantel AG, and Juchau MR (1991) Human embryonic cytochrome P450S: phenoxazone ethers as probes for expression of functional isoforms during organogenesis. *Biochemical pharmacology* **42:**2377-2385.

Miyagi SJ, Milne AM, Coughtrie MW, and Collier AC (2012) Neonatal development of hepatic UGT1A9: implications of pediatric pharmacokinetics. *Drug Metab Dispos* **40:**1321-1327.

Naraharisetti SB, Lin YS, Rieder MJ, Marciante KD, Psaty BM, Thummel KE, and Totah RA (2010) Human liver expression of CYP2C8: gender, age, and genotype effects. *Drug Metab Dispos* **38:**889-893.

Pelkonen O and Hakkola J (2008) Cytochromes P450, induction and cholesterol--what are the connections? *Eur J Clin Pharmacol* **64:**837-839.

Ross MK, Borazjani A, Edwards CC, and Potter PM (2006) Hydrolytic metabolism of pyrethroids by human and other mammalian carboxylesterases. *Biochemical pharmacology* **71:**657-669.

Rostami-Hodjegan A and Tucker GT (2007) Simulation and prediction of in vivo drug metabolism in human populations from in vitro data. *Nature reviews Drug discovery* **6:**140-148.

Schettgen T, Heudorf U, Drexler H, and Angerer J (2002) Pyrethroid exposure of the general population-is this due to diet. *Toxicol Lett* **134:**141-145.

Scollon EJ, Starr JM, Godin SJ, DeVito MJ, and Hughes MF (2009) In vitro metabolism of pyrethroid pesticides by rat and human hepatic microsomes and cytochrome p450 isoforms. *Drug Metab Dispos* **37:**221-228.

Sheets LP, Doherty JD, Law MW, Reiter LW, and Crofton KM (1994) Age-dependent differences in the susceptibility of rats to deltamethrin. *Toxicology and applied pharmacology* **126:**186-190.

Sonnier M and Cresteil T (1998) Delayed ontogenesis of CYP1A2 in the human liver. *European journal of biochemistry / FEBS* **251:**893-898.

Soyama A, Hanioka N, Saito Y, Murayama N, Ando M, Ozawa S, and Sawada J (2002) Amiodarone N-deethylation by CYP2C8 and its variants, CYP2C8*3 and CYP2C8 P404A. *Pharmacology & toxicology* **91:**174-178.

Soyama A, Saito Y, Hanioka N, Murayama N, Nakajima O, Katori N, Ishida S, Sai K, Ozawa S, and Sawada JI (2001) Non-synonymous single nucleotide alterations found in the CYP2C8 gene result in reduced in vitro paclitaxel metabolism. *Biological & pharmaceutical bulletin* **24:**1427-1430.

Stevens JC, Hines RN, Gu C, Koukouritaki SB, Manro JR, Tandler PJ, and Zaya MJ (2003) Developmental expression of the major human hepatic CYP3A enzymes. *The Journal of pharmacology and experimental therapeutics* **307:**573-582.

Totah RA and Rettie AE (2005) Cytochrome P450 2C8: substrates, inhibitors, pharmacogenetics, and clinical relevance. *Clinical pharmacology and therapeutics* **77:**341-352.

Yeo KR, Rostami-Hodjegan A, and Tucker G (2004) Abundance of cytochromes P450 in human liver: a meta-analysis, in: *British Journal of Clinical Pharmacology*, pp 687-688, BLACKWELL PUBLISHING LTD 9600 GARSINGTON RD, OXFORD OX4 2DG, OXON, ENGLAND.

Yoon M, Campbell JL, Andersen ME, and Clewell HJ (2012) Quantitative in vitro to in vivo extrapolation of cell-based toxicity assay results. *Critical reviews in toxicology* **42:**633-652.

Zanger UM and Schwab M (2013) Cytochrome P450 enzymes in drug metabolism: regulation of gene expression, enzyme activities, and impact of genetic variation. *Pharmacology & therapeutics* **138:**103-141.

FOOTNOTES

FUNDING INFORMATION: This study was supported by the Council for the Advancement of Pyrethroid Human Risk Assessment (CAPHRA), LLC.

DMD Fast Forward. Published on February 22, 2017 as DOI: 10.1124/dmd.116.074583
This article has not been copyedited and formatted. The final version may differ from this version.

FIGURE LEGENDS

Figure 1. Representative Western blots of CYP2C8 and 1A2 in human liver microsomal samples. (A) Lanes 1-6, purified recombinant CYP2C8 standards (1, 0.4, 0.13, 0.04, 0.015, 0.005 pmole); Lanes 7-12, tested human liver microsomal samples (lower bands, CYP2C8 5 or 10 μg each); Lane 13, commercial human pooled liver microsomal protein as quality control sample (lower band, 2 μg). (B) Lanes 1-6, purified recombinant CYP1A2 standards (0.04, 0.016, 0.0053, 0.0018, 0.0006, 0.0002 pmole); Lanes 7-12, tested human liver microsomal samples (5 or 10 μg each); Lane 13, commercial human pooled liver microsomal protein as quality control sample (2 μg).

Figure 2. (A) Human CYP2C8 protein content and age among microsomal liver fetal samples (N=60). PCA: post-conceptual age. (B) Human CYP2C8 and age among microsomal liver samples from the postnatal subjects less than one year of age (N=103). The added solid vertical line represents the 35 day time point selected by classification tree analysis and confirmed by statistical testing as appropriate age stratification.

DMD Fast Forward. Published on February 22, 2017 as DOI: 10.1124/dmd.116.074583
This article has not been copyedited and formatted. The final version may differ from this version.

Figure 3. (A) Age and human CYP2C8 content among microsomal liver samples from the subset of subjects from birth to 18 years (N=162). The added vertical lines represent the two nodes selected by classification tree analysis indicative of as appropriate age stratification: 35 days (solid line) and 11 years (dotted line). The 35-day age classification (solid line) was confirmed on Kruskal Wallis statistical testing followed by adjustment for multiple comparison using Dunn's post hoc test, whereas the 11-year age grouping (indicated by a dotted line) was not. (B) The relationship between human CYP2C8 content and age among postmortem microsomal liver samples from 222 human subjects from fetus to 18 years after birth. The vertical lines represent the two "cut points", age 35 days and 11 years (from right to left), selected by classification regression tree analysis as indicative of appropriate age groupings. PCA: post-conceptual age.

Figure 4. Summary of hepatic microsomal human CYP2C8 developmental expression pattern. CYP2C8 specific content as a function of age was grouped using classification tree analysis to minimize differences within while maximizing differences between age brackets. The resulting data are shown as box and whisker plots in which the horizontal bar represents median CYP2C8 content, boxes the upper and lower quartiles, and vertical bars the 5^{th} to 95^{th} percentiles. Outliers, defined as having specific contents outside 1.5 times the 25^{th} to 75^{th} precentiles, are shown as open circles. The youngest age group differed significantly from the other two groups ($p<0.0001$, each comparison, Kruskal-Wallis testing), whereas the middle age group did not differ from the oldest group ($p>0.05$).

Figure 5. (A) Age and human CYP1A2 among fetal hepatic microsomal samples (N=60). PCA: post-conceptual age. (B) Age and human CYP1A2 among microsomal liver samples from postnatal subjects less than 1 year of age (N=103).

Figure 6. (A) Human CYP1A2 content and age among microsomal liver samples from the subset of postnatal subjects from birth to 18 years (N=162). The added solid vertical line represents the 15-month time point selected by classification tree analysis and confirmed by statistical testing as appropriate age stratification. (B) The relationship between human CYP1A2 and age in postmortem microsomal liver samples from 222 human subjects from eight weeks gestation to 18 years after birth. The vertical line represents the time point selected by classification regression tree analysis as indicative of appropriate age groupings (15 months). PCA: post-conceptual age.

Figure 7. Summary of microsomal human CYP1A2 developmental expression pattern. CYP1A2 specific content as a function of age was grouped using classification tree analysis to minimize differences within while maximizing differences between age brackets. The resulting statistically significant data are shown as box and whisker plots in which the horizontal bar represents median CYP1A2 content, boxes the upper and lower quartiles, and vertical bars the 5^{th} to 95^{th} percentiles. Outliers, defined as having specific contents outside 1.5 times the 25^{th} to 75^{th} precentiles, are shown as open circles. The younger age group differed significantly from the other age group ($p<0.0001$, Mann-Whitney testing).

Figure 8. (A) The relationship between microsomal CYP2C8 and CYP2C9 content (linear regression line: $y=0.292 x +8.432$, $r^2=0.193$, $p<0.001$). (B) The relationship between microsomal CYP2C8 and CYP2C19 content (linear regression line: $y=0.269 x +4.825$, $r^2=0.321$, $p<0.001$). (C) The relationship between microsomal CYP2C8 and CYP3A4 content (linear regression line: $y=0.414 x +2.947$, $r^2=0.304$, $p<0.001$). Also, shown is the line of identity, dotted line.

DMD Fast Forward. Published on February 22, 2017 as DOI: 10.1124/dmd.116.074583
This article has not been copyedited and formatted. The final version may differ from this version.

Table 1. Demographics of Tissue Sample Donors

Variable		Median	Range
Age at death	All samples (PCA[a], weeks)	48.00	8.14 - 962.29
	Fetal samples only (PCA[a], weeks)	20.00	8.14 - 41.00
	Postnatal samples only (mos)	3.67	0.033 - 215.20
Postmortem Interval (hrs)		17	1-41
		N	**% of Total**
Sex	Male	136	61
	Female	76	34
	Unknown	10	5
Ethnicity	Northern European	92	41
	African American	84	38
	Hispanic	19	9
	Asian	2	1
	Native American	1	0.5
	Biracial	1	0.5
	Unknown	23	10

[a]PCA: post-conceptual age

DMD Fast Forward. Published on February 22, 2017 as DOI: 10.1124/dmd.116.074583
This article has not been copyedited and formatted. The final version may differ from this version.

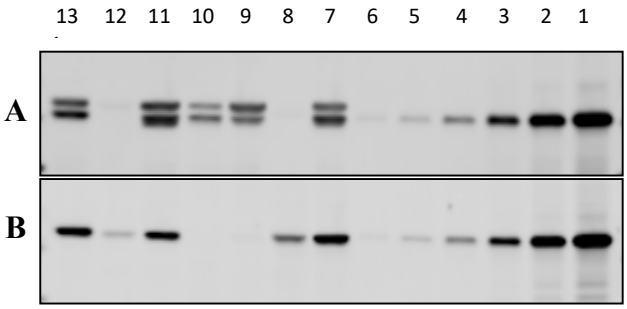

Figure 1

DMD Fast Forward. Published on February 22, 2017 as DOI: 10.1124/dmd.116.074583
This article has not been copyedited and formatted. The final version may differ from this version.

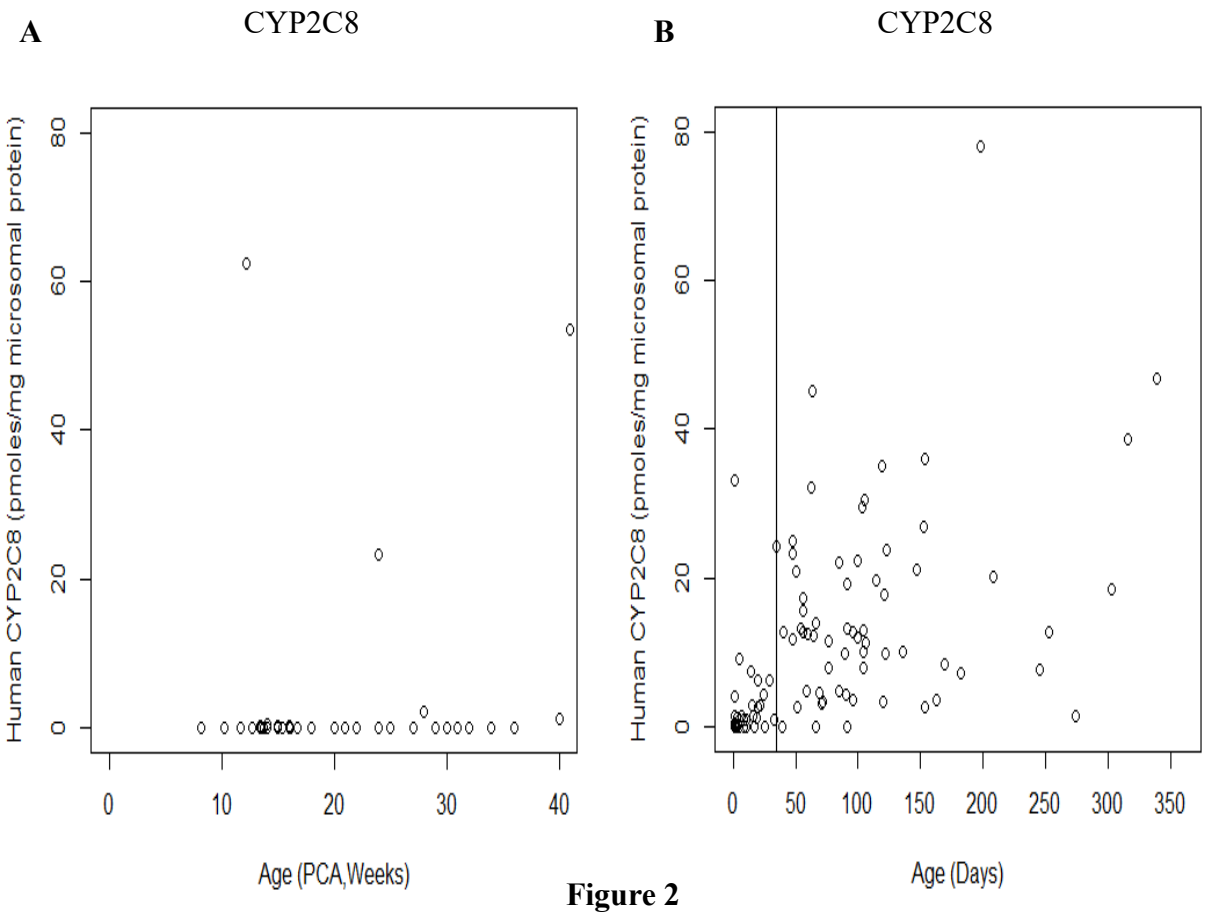

Figure 2

DMD Fast Forward. Published on February 22, 2017 as DOI: 10.1124/dmd.116.074583
This article has not been copyedited and formatted. The final version may differ from this version.

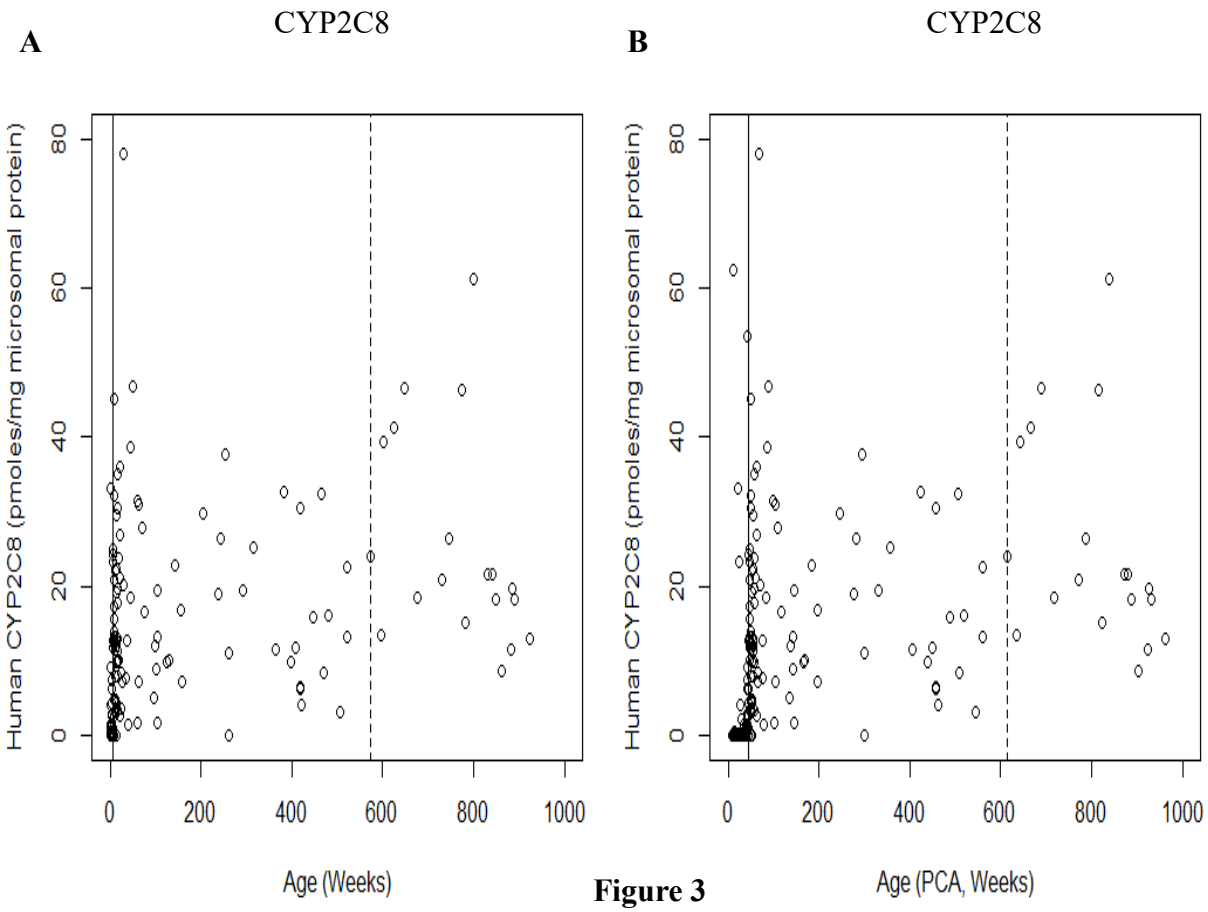

Figure 3

DMD Fast Forward. Published on February 22, 2017 as DOI: 10.1124/dmd.116.074583
This article has not been copyedited and formatted. The final version may differ from this version.

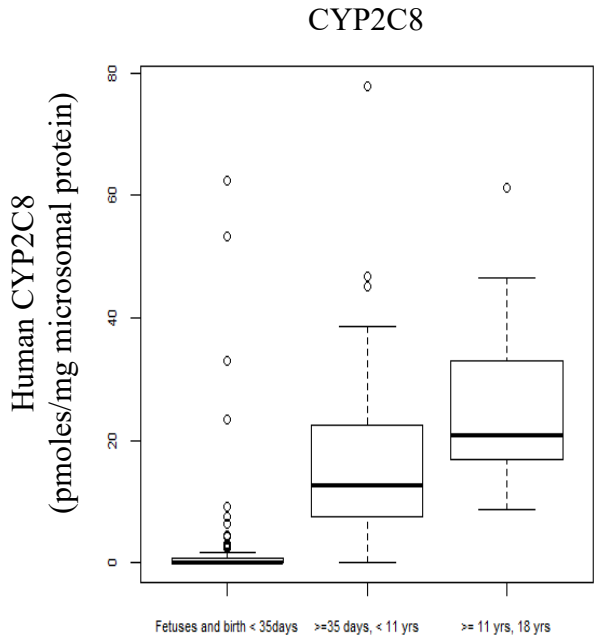

Figure 4

DMD Fast Forward. Published on February 22, 2017 as DOI: 10.1124/dmd.116.074583
This article has not been copyedited and formatted. The final version may differ from this version.

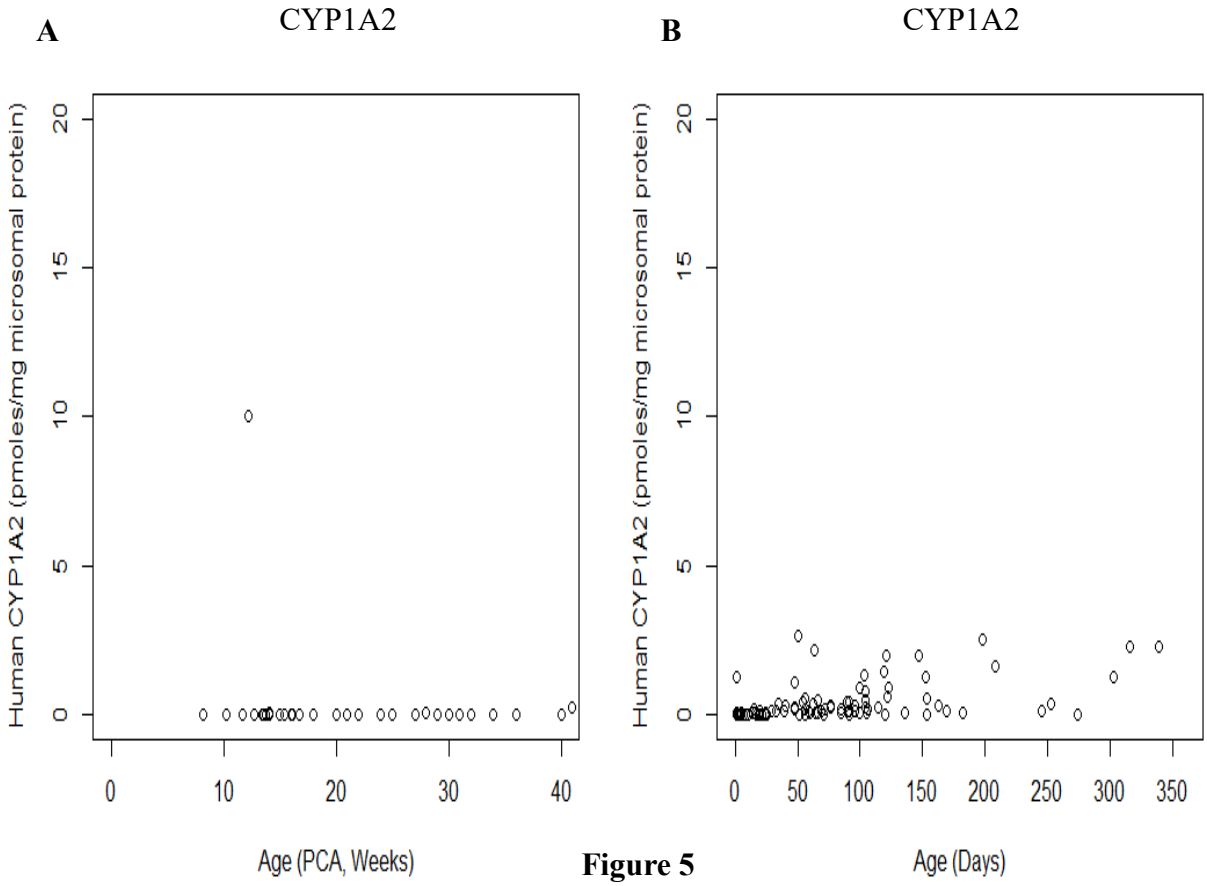

Figure 5

DMD Fast Forward. Published on February 22, 2017 as DOI: 10.1124/dmd.116.074583
This article has not been copyedited and formatted. The final version may differ from this version.

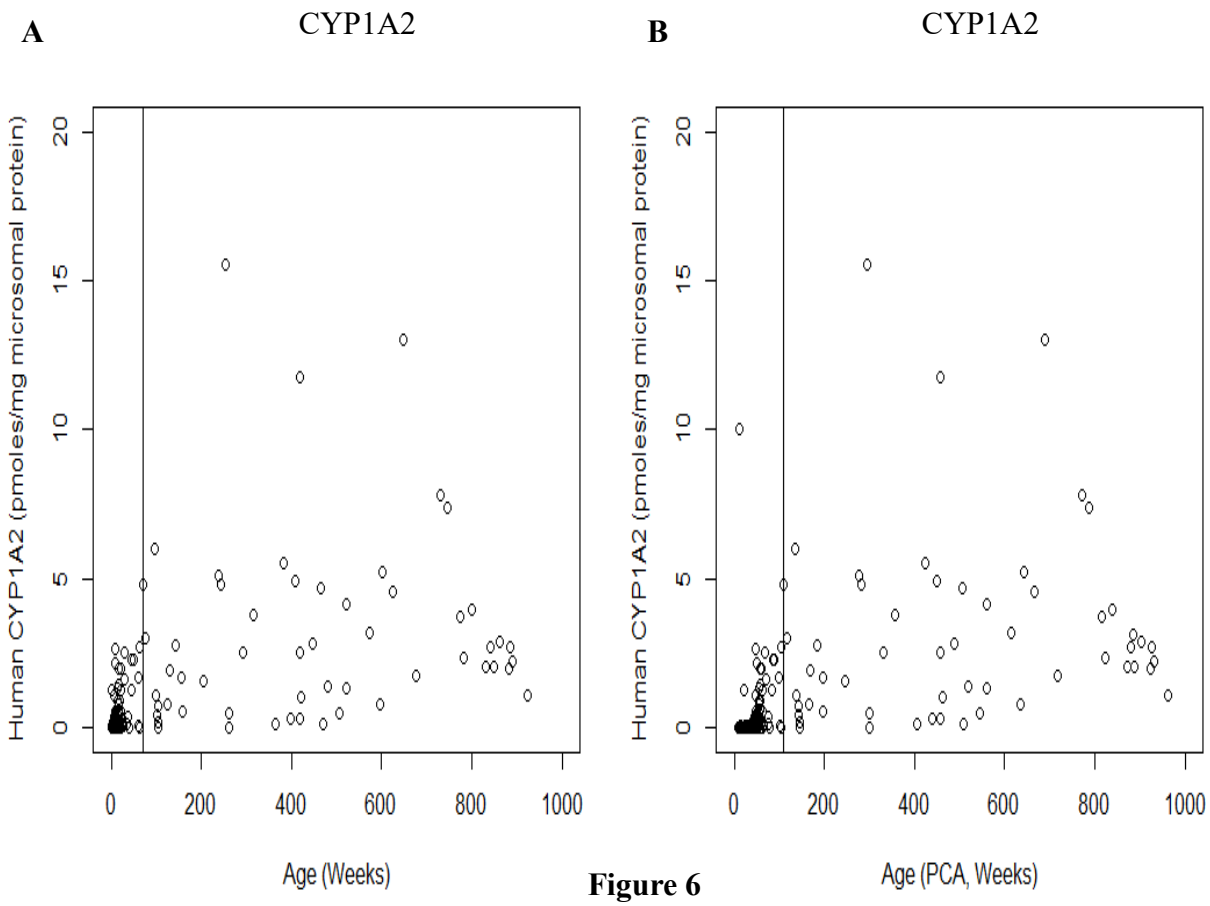

Figure 6

DMD Fast Forward. Published on February 22, 2017 as DOI: 10.1124/dmd.116.074583
This article has not been copyedited and formatted. The final version may differ from this version.

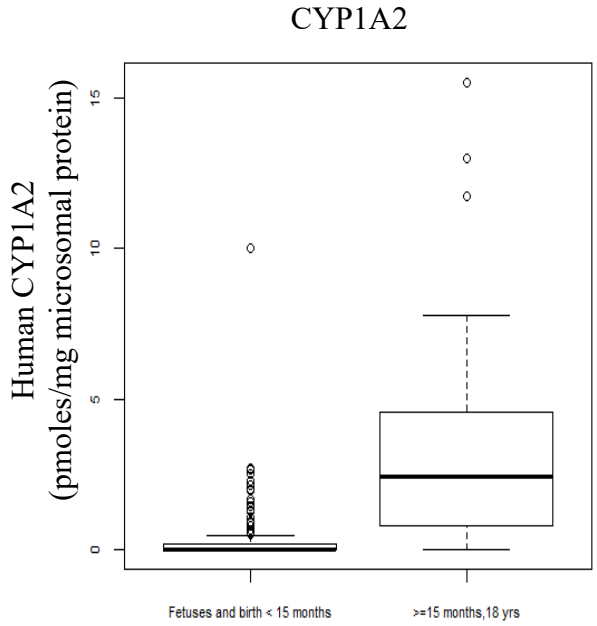

Figure 7

DMD Fast Forward. Published on February 22, 2017 as DOI: 10.1124/dmd.116.074583
This article has not been copyedited and formatted. The final version may differ from this version.

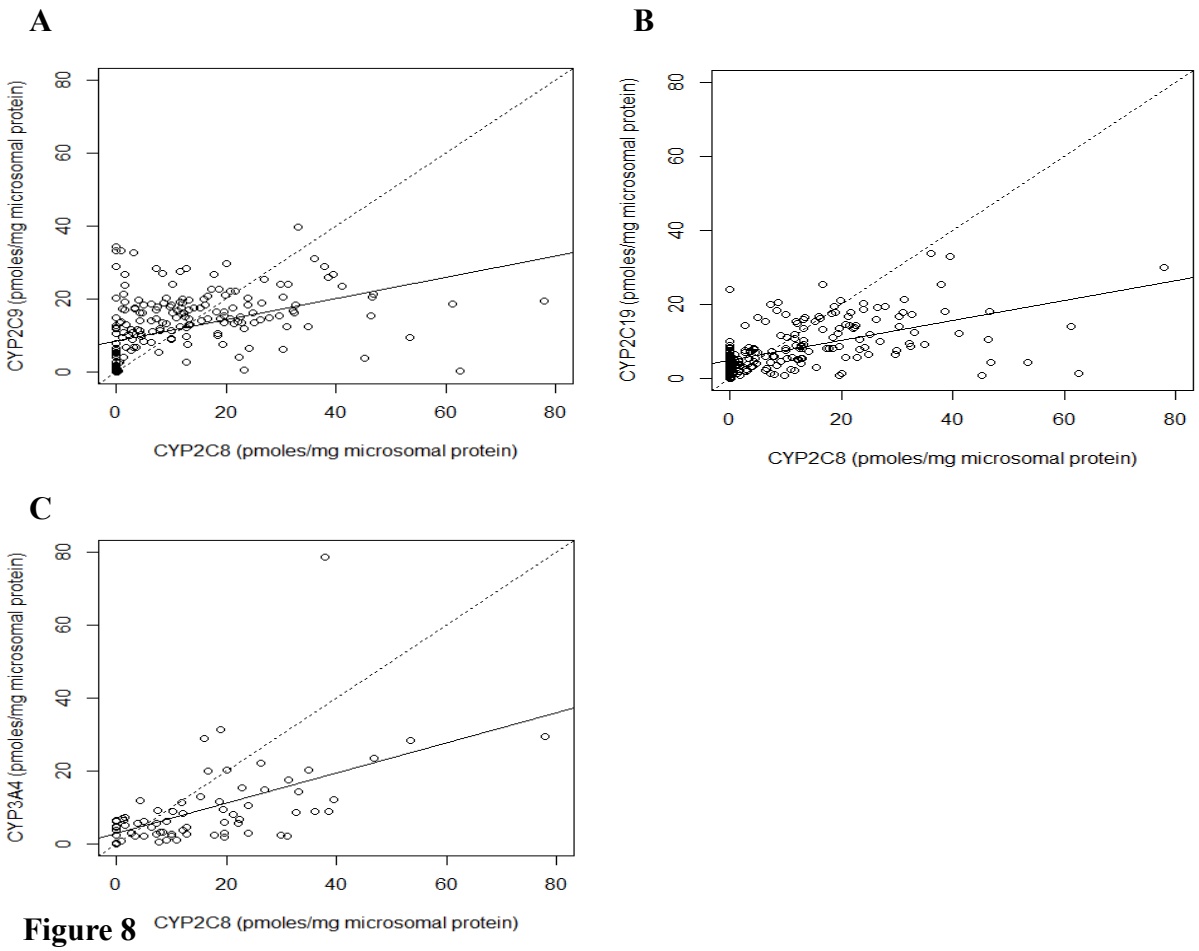

Figure 8