Cytochrome P450 and Flavin-containing Monooxygenase Families: Age-Dependent Differences in Expression and Functional Activity

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Abstract

Background: Age-dependent differences in pharmacokinetics exist for metabolically cleared medications. Differential contributions in the cytochrome P450 3A (CYP3A), CYP2C, and flavin-containing monooxygenases (FMO) families play an important role in the metabolic clearance of a large number of drugs administered to children.

Methods: Unlike previous semi-quantitative characterization of age-dependent changes in the expression of genes and proteins (Western Analysis), this study quantifies both gene and absolute protein expression in the same fetal, pediatric and adult hepatic tissue. Expression was then correlated with the corresponding functional activities in the same samples.

Results: CYP3A and FMO families showed a distinct switch from fetal (CYP3A7, FMO1) to adult isoforms (CYP3A4, FMO3) at birth, while CYP2C9 showed a linear maturation from birth into adulthood. In contrast, analysis of CYP2C19 revealed higher expression and catalytic efficiency in pediatric samples compared to fetal and adult samples. Further, CYP3A and FMO enzymes exhibited an unexpected higher functional activity in fetal samples not entirely explained by protein expression.

Conclusions: These surprising findings suggest that CYP and FMO enzymes may encounter development-related differences in their micro environments that can influence the enzyme activity in addition to protein expression levels.

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Introduction

For nearly 75% of the top 200 drugs on the market, hepatic clearance is the predominant clearance mechanism, and over three-quarters of hepatic clearance is due to phase I drug metabolizing enzymes (DMEs) (1). It is recognized that due to ontogeny of DMEs, many drugs exhibit differential pharmacokinetics (PK) in children of different age group compared to adults. However, our ability to predict PK in children based on this knowledge is somewhat limited due to many factors that are under active investigation. In a study comparing the PK of 45 drugs, children aged 2–12 years old had a higher average clearance and lower half-life compared to adults owing to age-related differences in metabolic clearance (2). These PK differences provide a basis for higher weight-based dosing for many drugs (3–6), including voriconazole, a drug that exhibits nearly 3-fold higher clearance in children (2–10 years old) than in adults (7). Voriconazole is cleared predominantly by oxidative metabolism through cytochrome P450 (CYP) 3A4, CYP2C19, and flavin-containing monooxygenases (FMO) 3 (8, 9). Early studies examined age-dependent differences in the relative expression of major DMEs responsible for medications cleared by oxidative metabolism, where protein expression was assessed via Western blotting or differential metabolism (10–13). Western Blot studies are semi-quantitative, and are sometimes limited by difficulties in the selectivity and cross-reactivity of the reagents used, which is overcome by using quantitative LC/MS-based methods to assess protein expression. Of note, a unique trend of higher CYP2C19 expression in pediatric samples (ages <10) over that in the older children or adults was overlooked, leaving unanswered questions about how differences in expression affect the activity of the CYP2C family in different populations (10).

The aim of this study was to quantify CYP3A, CYP2C, and FMO gene and absolute protein expression (14–16) in fetal, pediatric and adult hepatic tissue, and to correlate the gene and protein expression values with the corresponding functional activities in the same samples. We believe that a higher degree of fidelity achieved by absolute quantitation of enzyme proteins and measuring activity and protein expression in the same samples will enable the use of in vitro metabolism data to explore the knowledge gaps that limit current attempts to determine the factors responsible for observed differences in metabolic clearance for certain drugs among age groups spanning birth to adulthood.

Materials and Methods

Chemicals, Reagents, and Sample Selection

Testosterone, 6-β OH-testosterone, S-mephenytoin, 4'-OH S-mephenytoin, diclofenac, 4'-OH diclofenac, cimetidine, NADPH, and voriconazole were purchased from Sigma-Aldrich (St. Louis, MO). Cimetidine sulfoxide was purchased from Abcam (Cambridge, MA). Liver tissues from adult (>18 years old, median 39 years), pediatric (aged 1.9 months-10 years, median 1.5 years), and fetal (15–29 weeks gestation, median 16 weeks) donors were obtained from the NICHD Brain and Tissue Bank for Developmental Disorders (Contract #HHSN275200900011C, Ref. No. NO1-HD-9–0011; Baltimore, MD) under an approved UNC-Chapel Hill IRB. Fetal samples were obtained from terminated pregnancies. Pediatric tissues and adult tissues were from healthy donors (e.g. cause of death from trauma or

sudden infant death syndrome) or donors with no known hepatic dysfunction which could affect results. Females represented 2 out of the 7 fetal samples, 5 out of 16 pediatric samples (with 3 samples of unknown gender), and 6 out of 10 adult samples. Full donor information is listed in Supplemental Table S1.

Preparation of Microsomes

Human liver microsomes (HLM) were prepared as previously reported (17). Briefly, 0.5 mg of hepatic tissue was homogenized in a buffer containing 50 mM Tris-HCl, 1 mM EDTA, and 154 mM KCl with protease inhibitors (pH 7.4). After homogenization, samples were centrifuged at $10,000 \times$ g for 30 minutes, the supernatant was collected, and this step was repeated. The supernatant was mixed with a second buffer containing 100 mM sodium pyrophosphate and 0.1 mM EDTA (pH 7.4) and then centrifuged at $100,000 \times$ g for 70 minutes. This step was repeated one final time. The resulting pellet was then resuspended in a buffer containing 10 mM potassium phosphate, 1 mM EDTA, and 20% glycerol (pH 7.4). Microsomal protein concentration was measured using a BCA assay. HLM were stored at 20 μL aliquots at $-80°C$.

Gene & Protein Expression

Liver tissue cells (25mg) were lysed using Qiazol (Qiagen), sonicated until completely homogenized, and then centrifuged at $10,000 \times$ g for 12 minutes. Phase separation and binding of the mRNA was completed using a Qiagen RNEasy Kit. RNA purity and concentration was measured on a NanoDrop 2000 UV-Vis Spectrophotometer (Thermo Scientific; Wilmington, DE) and RNA was diluted to 500 ng/μL for each sample. To ensure sample purity, only samples with an A_{260}/A_{280} ratio between 1.9–2.1 were evaluated for gene expression. The isolated RNA was mixed with iScript™ cDNA Synthesis Kit (Biorad) and incubated at $25°C \times 5$ min, $42°C \times 30$ min, and then $85°C \times 5$ min to synthesize cDNA. The cDNA was mixed with iTaq™ Supermix (BioRad) and TaqMan Gene Expression Assay for CYP3A4, 3A5, 3A7, 2C9, 2C19, FMO1, FMO3 and 18s (Applied Bio-systems). Expression for each enzyme gene was measured (quantitative rt-PCR) and normalized to 18s rRNA. For protein quantitation of CYP3A and CYP2C families (signature peptide sequences shown in Supplemental Table S2), HLM were subjected to LC-MRM-based targeted quantitative proteomic analysis using recombinant proteins (Supersomes™; Corning Gentest, Woburn, MA) of known concentration as calibration standards (14, 16, 18). A more detailed description of the targeted quantitative proteomic methods was previously published (16, 19).

Functional Activity

Six samples from each population (fetal, pediatric, and adult) were chosen to determine differences in functional activity. Samples were selected at random with no prior knowledge about any enzyme activity of the samples. However, our random selection was limited by amount of tissue available. The HLM (0.1 mg/mL) or recombinant enzyme (0.05 mg/mL) along with the probe substrate of each respective enzyme (Table 1) were warned in phosphate buffer (pH 7.4) containing $MgCl_2$ (3 mM) at 37°C for 5 minutes. Functional activity reactions were initiated with the addition of NADPH (1 mM) and incubated up to 60 min in triplicate. Aliquots (100 μL) of samples were taken at specified time points, quenched

with 300 μL methanol containing internal standard (0.1 μM of either voriconazole for the CYP assays or phenformin for the FMO assay). Samples were then centrifuged at 10,000g × 10 min and analyzed by LC-MS/MS.

Metabolite Analysis

The LC-MS/MS system consisted of mobile phase (A) 0.1% formic acid in water and (B) 0.1% formic acid in methanol with a total flow rate of 0.6 mL/min. A Sciex API-4000 triple quadrupole mass spectrometer was used with a Zorbax SB-C18 column (2.1 × 50 mm, 5 μm). Samples were ionized using an ESI ion source and, following the injection of 10 μL of supernatant, positive ions were monitored at the following MRM Q1/Q3 transitions (*m/z*): 235.2→150.2 for 4'-OH S-mephenytoin, 312.3→230.2 for 4'-OH diclofenac and 350.2→127.2 for voriconazole. For the testosterone assay, samples were ionized using an APCI ion source and monitored at the following MRM Q1/Q3 transitions (*m/z*): 289.2→97.1 for testosterone, 305.1→269.3 for 6β-OH testosterone and 350.2→127.2 voriconazole.(16, 20, 21)

Statistical Analysis

Differences between multiple populations were analyzed using Kruskal Wallis and Dunn's posthoc test to correct for multiple comparisons and tests between two groups were analyzed using Wilcoxon signed-rank test. The linearity of maturation profiles and correlation analyses were determined by fitting a linear regression curve and 95% confidence intervals to the model. For all analyses, a p-value less than 0.05 was considered significant.

Results

CYP3A, CYP2C, and FMO Expression

Relative gene expression trends of the three major CYP3A enzymes (Figure 1a) corresponded well to the trends in protein expression (Figure 2). However, statistical significance for the differences in expression could only be achieved for proteins due to high variability observed in gene expression. As expected, fetal samples had very high expression of CYP3A7 protein (0.535 nmol/mg) with a corresponding low expression of CYP3A4 protein (0.0003 nmol/mg). During maturation, CYP3A7 protein levels decreased in the pediatric and adult populations (0.051- and 0.019 nmol/mg, respectively; Figure 2a). The adult CYP3A7 expression is slightly skewed by a high expression in one sample from an 80 year old patient, which, if excluded, reduces the adult CYP3A7 expression to 0.0052 nmol/mg. On the other hand, CYP3A4 levels increased from undetectable levels in the fetal period until it reached adult levels (0.069–0.078 nmol/mg) around 1 year of age. Abundance of CYP3A7 was over 10-fold higher and abundance of CYP3A4 was over 200-fold lower in the fetal tissues compared to either the pediatric or adult tissues (p < 0.01; Figure 2d). This switch from CYP3A7 as the predominant isoform in fetal tissues to CYP3A4 in adult tissues was seen previously in the literature (13). CYP3A5 remains relatively stable (0.001–0.005 nmol/mg) across all three groups with no statistically significant difference among the three populations. Taken together, gene and protein expression data demonstrate that there is a clear transition of the predominant CYP3A isoform from the fetal population (CYP3A7) into adulthood (CYP3A4). These results generally confirm the pattern of age-

related changes in the expression of CYP 3A enzymes that is reported previously (12, 13, 22).

Expression of both CYP2C9 and CYP2C19 genes was low to undetectable in fetal samples, with approximately a 100-fold lower expression compared to the pediatric and adult samples (Figure 1b). No significant differences were observed in CYP2C9 or CYP2C19 gene expression between adults and children. However, there was a trend towards higher CYP2C19 gene expression in the pediatric population compared to the adult population (p = 0.062). In contrast, protein expression of CYP2C9 protein increased linearly over time ($r^2 = 0.31$; $p < 0.0001$) and adult level expression was reached in the pediatric population (Figure 2b). Overall, expression of CYP2C9 protein corresponded to its gene expression (Figure 2e); fetal tissue expressed significantly lower levels (0.001 nmol/mg) of CYP2C9 protein compared to pediatric and adult tissues (0.052 and 0.054 nmol/mg, respectively; $p < 0.001$).

In contrast to the linear maturation profile of CYP2C9 protein, expression of CYP2C19 protein peaked during the pediatric period (Figure 2b). CYP2C19 protein expression in pediatric tissues was over 2-fold higher compared to adult tissues (0.0093 nmol/mg versus 0.0043 nmol/mg; p=0.046). This confirms our previous observation of higher CYP2C19 protein expression in the hepatic tissue of children (2 to 10 years old) than in the adult hepatic tissue; however, since the previous results were based on Western blot analysis, and were semi-quantitative at best (21), the present results provide a more definitive estimate of the differences in the hepatic CYP2C29 protein expression between children and adult.

FMO gene expression exhibited high variability, which limited the detection of significant differences between populations or isoforms, but the trends corresponded well to those seen with protein expression. As expected, relatively high protein expression of FMO1 was observed in fetal samples (0.007 nmol/mg) with a low expression of FMO3 (0.001 nmol/mg; Figure 2c). FMO1 expression was virtually undetectable in pediatric and adult samples (0.00002 nmol/mg in each population), confirming the notion that FMO1 is a fetal isoform (11). Because of the specificity of the protein expression assay and low variability in the protein expression data, a significant difference in the expression of FMO3 protein was observed among all three populations, with an increase from 0.001 to 0.02 to 0.037 nmol/mg in the fetal, pediatric, and adult samples, respectively ($p < 0.05$; Figure 2f). Thus, there was a clear ontogeny profile for FMO3 protein expression with a linear increase from the fetal period into adulthood ($r^2 = 0.52$; $p < 0.0001$). As seen in the CYP3A family, a distinct transition occurred from a predominant fetal isoform (FMO1) to an adult isoform (FMO3) after birth.

CYP3A, CYP2C, and FMO Enzyme Activities Normalized to Respective Protein Expression

The protein expression levels of the CYP and FMO enzymes were correlated to corresponding enzyme activity using enzyme-specific probe substrates. Testosterone was chosen as the probe substrate to assess functional activity of the CYP3A family in the fetal, pediatric and adult populations, and in recombinant CYP3A4, 3A5, and 3A7 enzymes (Figure 3). Metabolite formation was normalized to the total amount of CYP3A protein in each sample as measured by the targeted quantitative proteomic method, and a clear maturation trend was observed for the catalytic activity for oxidation of testosterone across

the three populations (1.2, 1.6, and 2.8 pmol/min/pmol 3A, respectively). This maturation profile was consistent with the formation of metabolite by the recombinant 3A4, 3A5 and 3A7 enzymes (3.9, 3.0, and 1.4 pmol/min/pmol 3A respectively).

Diclofenac and S-mephenytoin were used as probe substrates to assess how changes in protein expression with age affects the functional activities of CYP2C9 and CYP2C19, respectively. Formation of 4'-hydroxylation of diclofenac by fetal tissue, normalized to total microsomal protein (Figure 4a), was over 300-fold lower than that observed for the pediatric and adult samples (0.24 versus 96 versus 83 pmol/min/mg protein, respectively). After normalizing metabolite formation to the total CYP2C9 protein in each sample (Figure 4b), fetal samples still had much lower metabolic activity compared to pediatric and adult samples (0.2 versus 1.6 and 1.4 pmol/mg/pmol CYP2C9 respectively), with no significant difference in CYP2C9 activity between adult and pediatric populations.

CYP2C19 activity displayed a different maturation profile compared to CYP2C9. Consistent with nearly undetectable CYP2C19 protein levels in fetal tissue, formation of 4'-hydroxylation of S-mephenytoin by fetal samples was undetectable (Figure 4c). However, metabolic activity in pediatric samples was higher than that in adult samples when normalized to total microsomal protein (1.6 versus 0.51 pmol/min/mg protein respectively; $p < 0.04$; Figure 4c). *Even after normalizing for the higher expression of CYP2C19 protein in pediatric samples, a significant difference between the metabolite formation rates by the pediatric and adult enzyme (0.15 versus 0.079 pmol/min/pmol CYP2C19, respectively; $p < 0.01$) was observed.* Even after excluding the samples with the highest formation rates (which may be attributed to rapid metabolizers although this is not confirmed by genetic analysis), the difference between pediatric and adult samples retained statistical significance ($p < 0.04$). These results suggest that a yet unidentified cellular mechanism, in combination with higher CYP2C19 expression, confers higher metabolic activity in the pediatric population. Overall, CYP2C9 and CYP2C19 catalytic activities toward their respective probe substrates are significantly correlated with the abundance of each enzyme across the age groups (Figure 5).

Functional activity of FMO was assessed using cimetidine as a probe substrate. Formation of cimetidine sulfoxide by recombinant FMO1 was 2-fold higher than by recombinant FMO3. Fetal samples, which had a higher expression of FMO1, generated cimetidine sulfoxide at a rate 2-fold higher than that observed with pediatric and adult samples ($p < 0.003$; Figure 6a). Normalization of FMO functional activity to its abundance in the corresponding tissue revealed a surprising finding that catalytic activity of FMO is significantly higher ($p < 0.0001$) in fetal samples (0.20 pmol/min/pmol FMO) compared to pediatric and adult samples (0.005 and 0.01 pmol/min/pmol FMO, respectively; Figure 6b).

DISCUSSION

It is recognized that gene and protein expression of DMEs changes with age. While numerous studies have investigated these changes, the protein measurements have been done by Western Blot analysis, which is considered to be a semi-quantitative method. In the present study, we have determined protein expression quantitatively using an LC-MS/MS

method, and have related gene and protein expression to the catalytic activity of CYP3A, CYP2C, and FMO enzymes, normalized to the respective enzyme protein, in the same respective samples of the fetal, pediatric, and adult hepatic tissues. We acknowledge that the sample size is small and that range of fetal age considered was not exhaustive, and therefore the conclusions reached in this study should be verified in future studies with additional tissue samples in these age groups and expanded fetal age groups. However, our approach of relating quantitative protein expression measurements and enzyme activity in the same tissues provides reliable and self-consistent data on the metabolic changes due to the three enzyme families in the fetal, pediatric, and adult populations.

A comparison between gene and protein expression profiles indicate that gene expression can reveal relative protein expression trends among CYP isoforms within the same enzyme family. Despite this loose correlation, gene expression cannot be used as a surrogate for respective protein expression. For instance, adult CYP2C9 gene expression suggests a similar abundance to fetal CYP3A7, and a 2.5-fold higher expression compared to adult CYP3A4 gene. However, CYP3A protein quantification reveals higher expression compared to CYP2C9, which is in agreement with published reports (23). Differences in primer specificity used to quantify gene expression can alter results, and post-translational modifications can influence protein expression, thus leading to inaccurate predictions in PK parameters for pediatric drugs if gene expression is used as a measure of protein expression.

In regards to protein expression, traditional methods (eg Western blotting or differential metabolism) utilize antibodies that poses the likelihood of confounding the data due to cross-reactivity with other isoforms in the same family or measurement of functionally inactive apoproteins (19). Whereas, quantitative proteomic analysis provides enhanced accuracy of enzyme abundance since target peptides are selected from an isoform's specific peptide sequence (15). Therefore, the results presented here provide a more quantitative assessment of protein expression compared to previous studies that utilized Western blot or differential metabolism to determine isoform expression within the same family (10, 13, 24).

The quantitative determination of protein expression in fetal, pediatric, and adult hepatic tissues provided the opportunity to correlate protein expression with functional activities of the DMEs in these age groups. Previous studies and reviews have reported that CYP3A7 has a lower catalytic activity (as much as 17-fold) for testosterone 6β-hydroxylation compared to CYP3A4 (12, 13, 22). In contrast to previously reported results, we have observed that CYP3A7 testosterone metabolism is greater than that of CYP3A4 (25). This was missed in the previous studies because the activities were compared based on 6β-OH testosterone formation, which is not the major metabolite of CYP3A7 (25). The results show that the specific activity (i.e. catalytic activity normalized to the enzyme protein) of CYP3A recombinant enzymes measured in supersomes is higher than the corresponding specific activity measured in HLM. This could be due to differences in NADPH coupling to the CYP enzyme or different lipid environment, yielding different substrate access to the enzyme.

Interestingly, protein expression of CYP2C19 was higher (nearly 2-fold) in pediatric liver samples compared to adult liver samples. This distinctive trend was observed in previous reports where CYP2C19 specific activity was approximately 20% higher in children aged

5 months – 10 years compared to children 10–18 years old (10). Similar results regarding CYP2C19 activity differences were reported by Yanni et al. who also compared CYP2C19 protein expression using Western blot analysis. They demonstrated a trend for higher expression in children (2–12 years old) compared to adults (> 18 years old), but did not reach significance due to high variability (21). While neither of these reports could definitively establish higher pediatric CYP2C19 expression, the sensitivity of the LC-MS-based targeted quantitative proteomic assay to determine CYP2C19 expression decreased variability and provided definitive evidence of a significant difference in CYP2C19 protein expression between these two populations. Higher expression of CYP2C19 in children may explain, in part, the higher pediatric clearance of voriconazole compared to adults, and previous findings that CYP2C19 accounts for higher voriconazole metabolism in children *versus* adults (21, 26).

Surprisingly, when the catalytic activity of CYP2C19 was normalized to CYP2C19 protein expression, the turnover rate of the CYP2C19 probe S-mephenytoin was higher for pediatric HLM compared to adult HLM, suggesting higher catalytic efficiency of CYP2C19 in children. This significant difference remained even after excluding the samples with the highest formation rate, which could be due to CYP2C19 polymorphisms. One likely explanation for this may be a better coupling of NADPH cytochrome P450 oxidoreductase or cytochrome b5 to CYP2C19 either due to a better oxidoreductase/cytochrome b5 to CYP2C19 stoichiometry or due to different lipid environment. Structure, function, and genetic polymorphisms of NADPH cytochrome P450 oxidoreductase have been shown to affect drug metabolism of compounds such as steroids and midazolam (27–30). Importantly, specific variants of NADPH cytochrome P450 oxidoreductase increased the metabolic activity of both CYP2C19 and CYP1A2 (31). Since cytochrome b5 can augment cytochrome P450 activity by an alternative or additional electron transfer process or by allosteric activation (32), it is conceivable that higher specific activity of CYP2C19 in children may be caused by cytochrome b5. Another potential factor affecting catalytic efficiency of CYP2C19 is the lipid composition of the membrane within the endoplasmic reticulum. Accessibility of a cytochrome P450 active site requires a substrate to traverse the lipid membrane in which the enzyme is embedded, so the same cytochrome P450 enzyme could catalyze the same substrate with different efficiencies in two different microsomal samples if lipid composition differs. Since it is known that fat composition changes during development, it is conceivable that the composition of cell membranes in children may be different from that in adults. Irrespective of the mechanism, higher catalytic efficiency of CYP2C19 in pediatric liver, in addition to higher CYP2C19 protein expression, could be responsible for the higher contribution of CYP2C19 toward voriconazole metabolism and its higher clearance in children compared to adults, which could not be accounted for by 2-fold greater liver to body mass ratio in children compared to adults. The higher catalytic efficiency of CYP2C19 in pediatric compared to adult HLM is reported for the first time in this study. Hence, we suggest that age-related differences in the molecular and biophysical determinants of the catalytic efficiency of DMEs should be further investigated. Future research should derive PK parameters of drugs should using PBPK models that assume age-related changes in the catalytic efficiency of the CYP enzymes responsible for their

metabolism and compared to the parameters derived using models that fix the catalytic efficiency of the CYP enzymes across different age groups.

Finally, functional FMO activity of fetal, pediatric, and adult HLM was assessed using cimetidine as a probe. Cimetidine has been studied in preclinical species as well as in human clinical studies, and the evidence thus far suggests that FMO3 is the major metabolizing enzyme of cimetidine in adult humans (33, 34). Interestingly, the results in this study show that the rate of cimetidine sulfoxide formation by fetal HLM and recombinant FMO1, normalized to FMO1 protein, was ~2-fold higher than by pediatric and adult HLM and recombinant FMO3, normalized to FMO3 protein. As was seen with CYP3A, the switch from FMO1 to FMO3 is linked to parturition rather than to gestational age (11, 35). These maturational differences in activity could also be attributed to differences in microenvironment or a coupling of the FMO proteins to their co-factor, FAD (19) and are potentially regulated by hormonal changes that occur during gestation and at birth.

Other studies have also investigated the age-dependent relationship between gene expression, protein expression, and enzyme activity. In one study by Sadler et al., which covered a larger gestational ages than are considered in the present study, the authors note that it is crucial to determine pediatric activity as it is not always correlated to expression (36). The present study reaches a similar conclusion that with the growing importance of physiologically-based modeling in pediatric clinical pharmacology, it is essential to determine the activity of each enzyme in a specific age-bracket in order to accurately simulate pediatric PK.

One limitation of our study is the small sample size due to limited availability of fetal and pediatric tissues, which is not unexpected in pediatric studies. On the other hand, selective and specific analytical methods were utilized to reduce variability in the measurement of protein expression. In addition, only samples with acceptable quality mRNA were included in gene expression analysis and quality and intra-day variability was assessed for protein expression. We observe that protein expression data for all three families that are reported in this study are consistent with those reported using semiquatitative methods in previous literature reports. We acknowledge that the small sample size limits the detection of differences in the expression and/or function of DMEs due to polymorphisms. Since clinically significant polymorphisms have been documented for CYP2C19, CYP2C9, FMO, and CYP3A5 (7, 24, 37–39), further research is warranted to understand the interaction of polymorphisms, ontogeny, expression, and activity.

Overall, the CYP3A and CYP2C families account for 60% of hepatic enzymes and metabolize over 70% of drugs currently on the market (1, 23). While the FMO family exhibits lower expression, it plays a pivotal role in regulating reactive oxygen species and metabolizing nitrogen- and sulfur-containing compounds (35). The unique differences in enzyme contributions that exist among the CYP3A, CYP2C and FMO families during development affect pediatric PK. While age-dependent changes in hepatic expression and activity have been examined previously, this is the first study to measure these changes based on targeted quantitative proteomics using the same samples. In addition, this is the first study, to our knowledge, that compares against one another and assesses three common

techniques utilized to understand how differences in ontogeny can affect dosing in children and provides concrete evidence for the optimal method to utilize in future research. Most importantly, this research lays the groundwork for future investigations into the age-related biochemical and molecular differences as determinants of DME, which can be simulated using PBPK modeling.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Statement of Financial Support

References

1. Wienkers LC, Heath TG 2005 Predicting in vivo drug interactions from in vitro drug discovery data. Nat Rev Drug Discov 4:825–833. [PubMed: 16224454]

2. Ginsberg G, Hattis D, Sonawane B, et al. 2002 Evaluation of child/adult pharmacokinetic differences from a database derived from the therapeutic drug literature. Toxicol Sci 66:185–200. [PubMed: 11896285]

3. Andersson T, Hassall E, Lundborg P, et al. 2000 Pharmacokinetics of orally administered omeprazole in children. International Pediatric Omeprazole Pharmacokinetic Group. Am J Gastroenterol 95:3101–3106. [PubMed: 11095324]

4. Kanamori M, Takahashi H, Echizen H 2002 Developmental changes in the liver weight- and body weight-normalized clearance of theophylline, phenytoin and cyclosporine in children. Int J Clin Pharmacol Ther 40:485–492. [PubMed: 12698985]

5. Litalien C, Theoret Y, Faure C 2005 Pharmacokinetics of proton pump inhibitors in children. Clin Pharmacokinet 44:441–466. [PubMed: 15871633]

6. Tran A, Rey E, Pons G, et al. 2002 Pharmacokinetic-pharmacodynamic study of oral lansoprazole in children. Clin Pharmacol Ther 71:359–367. [PubMed: 12011821]

7. Leveque D, Nivoix Y, Jehl F, et al. 2006 Clinical pharmacokinetics of voriconazole. Int J Antimicrob Agents 27:274–284. [PubMed: 16563707]

8. Hyland R, Jones BC, Smith DA 2003 Identification of the cytochrome P450 enzymes involved in the N-oxidation of voriconazole. Drug Metab Dispos 31:540–547. [PubMed: 12695341]

9. Yanni SB, Annaert PP, Augustijns P, et al. 2008 Role of flavin-containing monooxygenase in oxidative metabolism of voriconazole by human liver microsomes. Drug Metab Dispos 36:1119–1125. [PubMed: 18362161]

10. Koukouritaki SB, Manro JR, Marsh SA, et al. 2004 Developmental expression of human hepatic CYP2C9 and CYP2C19. J Pharmacol Exp Ther 308:965–974. [PubMed: 14634042]

11. Koukouritaki SB, Simpson P, Yeung CK, et al. 2002 Human hepatic flavin-containing monooxygenases 1 (FMO1) and 3 (FMO3) developmental expression. Pediatr Res 51:236–243. [PubMed: 11809920]

12. Lacroix D, Sonnier M, Moncion A, et al. 1997 Expression of CYP3A in the human liver-Eevidence that the shift between CYP3A7 and CYP3A4 occurs immediately after birth. Eur J Biochem 247:625–634. [PubMed: 9266706]

13. Stevens JC, Hines RN, Gu C, et al. 2003 Developmental expression of the major human hepatic CYP3A enzymes. J Pharmacol Exp Ther 307:573–582. [PubMed: 12975492]

14. Michaels S, Wang MZ 2014 The revised human liver cytochrome P450 "Pie": absolute protein quantification of CYP4F and CYP3A enzymes using targeted quantitative proteomics. Drug Metab Dispos 42:1241–1251. [PubMed: 24816681]

15. Ohtsuki S, Uchida Y, Kubo Y, et al. 2011 Quantitative targeted absolute proteomics-based ADME research as a new path to drug discovery and development: methodology, advantages, strategy, and prospects. J Pharm Sci 100:3547–3559. [PubMed: 21560129]

16. Wang MZ, Wu JQ, Dennison JB, et al. 2008 A gel-free MS-based quantitative proteomic approach accurately measures cytochrome P450 protein concentrations in human liver microsomes. Proteomics 8:4186–4196. [PubMed: 18792928]

17. Fisher CD, Lickteig AJ, Augustine LM, et al. 2009 Hepatic cytochrome P450 enzyme alterations in humans with progressive stages of nonalcoholic fatty liver disease. Drug Metab Dispos 37:2087–2094. [PubMed: 19651758]

18. Kawakami H, Ohtsuki S, Kamiie J, et al. 2011 Simultaneous absolute quantification of 11 cytochrome P450 isoforms in human liver microsomes by liquid chromatography tandem mass spectrometry with in silico target peptide selection. J Pharm Sci 100:341–352. [PubMed: 20564338]

19. Chen Y, Zane NR, Thakker DR, et al. 2015 Quantification of Flavin-containing Monooxygenases 1, 3 and 5 in Human Liver Microsomes by UPLC-MRM-based Targeted Quantitative Proteomics. Drug Metab Dispos Under Revision.

20. Daniele G, Fieu M, Joachim S, et al. 2016 Rapid analysis of diclofenac and some of its transformation products in the three-spined stickleback, Gasterosteus aculeatus, by liquid chromatography-tandem mass spectrometry. Anal Bioanal Chem 408:4435–4444. [PubMed: 27086017]

21. Yanni SB, Annaert PP, Augustijns P, et al. 2010 In vitro hepatic metabolism explains higher clearance of voriconazole in children versus adults: role of CYP2C19 and flavin-containing monooxygenase 3. Drug Metab Dispos 38:25–31. [PubMed: 19841059]

22. Ince I, Knibbe CA, Danhof M, et al. 2013 Developmental changes in the expression and function of cytochrome P450 3A isoforms: evidence from in vitro and in vivo investigations. Clin Pharmacokinet 52:333–345. [PubMed: 23463352]

23. Shimada T, Yamazaki H, Mimura M, et al. 1994 Interindividual variations in human liver cytochrome P-450 enzymes involved in the oxidation of drugs, carcinogens and toxic chemicals: studies with liver microsomes of 30 Japanese and 30 Caucasians. J Pharmacol Exp Ther 270:414–423. [PubMed: 8035341]

24. Koukouritaki SB, Poch MT, Cabacungan ET, et al. 2005 Discovery of novel flavin-containing monooxygenase 3 (FMO3) single nucleotide polymorphisms and functional analysis of upstream haplotype variants. Mol Pharmacol 68:383–392. [PubMed: 15858076]

25. Zane NR 2015 Predicting Pharmacokinetic Behavior and Dose of Sildenafil and Voriconazole in Neonatal and Pediatric Populations by In Vitro Metabolism and PBPK Modeling. Pharmaceutical Sciences. The University of North Carolina at Chapel Hill, ProQuest, p 183.

26. Zane NR, Thakker DR 2014 A Physiologically Based Pharmacokinetic Model for Voriconazole Disposition Predicts Intestinal First-pass Metabolism in Children. Clin Pharmacokinet 53:1171–1182. [PubMed: 25245942]

27. Chen X, Pan LQ, Naranmandura H, et al. 2012 Influence of various polymorphic variants of cytochrome P450 oxidoreductase (POR) on drug metabolic activity of CYP3A4 and CYP2B6. PLoS One 7:e38495. [PubMed: 22719896]

28. Oneda B, Crettol S, Jaquenoud Sirot E, et al. 2009 The P450 oxidoreductase genotype is associated with CYP3A activity in vivo as measured by the midazolam phenotyping test. Pharmacogenet Genomics 19:877–883. [PubMed: 19801957]

29. Pandey AV, Fluck CE 2013 NADPH P450 oxidoreductase: structure, function, and pathology of diseases. Pharmacol Ther 138:229–254. [PubMed: 23353702]

30. Pandey AV, Sproll P 2014 Pharmacogenomics of human P450 oxidoreductase. Front Pharmacol 5:103. [PubMed: 24847272]

31. Agrawal V, Huang N, Miller WL 2008 Pharmacogenetics of P450 oxidoreductase: effect of sequence variants on activities of CYP1A2 and CYP2C19. Pharmacogenet Genomics 18:569–576. [PubMed: 18551037]

32. Porter TD 2002 The roles of cytochrome b5 in cytochrome P450 reactions. J Biochem Mol Toxicol 16:311–316. [PubMed: 12481306]

33. Cashman JR, Park SB, Berkman CE, et al. 1995 Role of hepatic flavin-containing monooxygenase 3 in drug and chemical metabolism in adult humans. Chem Biol Interact 96:33–46. [PubMed: 7720103]

34. Cashman JR, Park SB, Yang ZC, et al. 1993 Chemical, enzymatic, and human enantioselective S-oxygenation of cimetidine. Drug Metab Dispos 21:587–597. [PubMed: 8104117]

35. Krueger SK, Williams DE 2005 Mammalian flavin-containing monooxygenases: structure/ function, genetic polymorphisms and role in drug metabolism. Pharmacol Ther 106:357–387. [PubMed: 15922018]

36. Sadler NC, Nandhikonda P, Webb-Robertson BJ, et al. 2016 Hepatic Cytochrome P450 Activity, Abundance, and Expression Throughout Human Development. Drug Metab Dispos 44:984–991. [PubMed: 27084891]

37. Weiss J, Ten Hoevel MM, Burhenne J, et al. 2009 CYP2C19 genotype is a major factor contributing to the highly variable pharmacokinetics of voriconazole. J Clin Pharmacol 49:196–204. [PubMed: 19033450]

38. Shirasaka Y, Chang SY, Grubb MF, et al. 2013 Effect of CYP3A5 expression on the inhibition of CYP3A-catalyzed drug metabolism: impact on modeling CYP3A-mediated drug-drug interactions. Drug Metab Dispos 41:1566–1574. [PubMed: 23723360]

39. Jacobson PA, Oetting WS, Brearley AM, et al. 2011 Novel polymorphisms associated with tacrolimus trough concentrations: results from a multicenter kidney transplant consortium. Transplantation 91:300–308. [PubMed: 21206424]
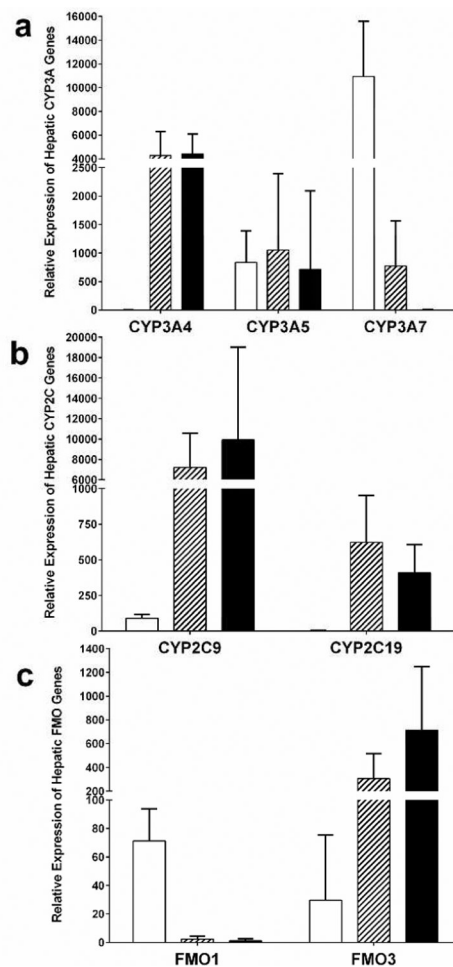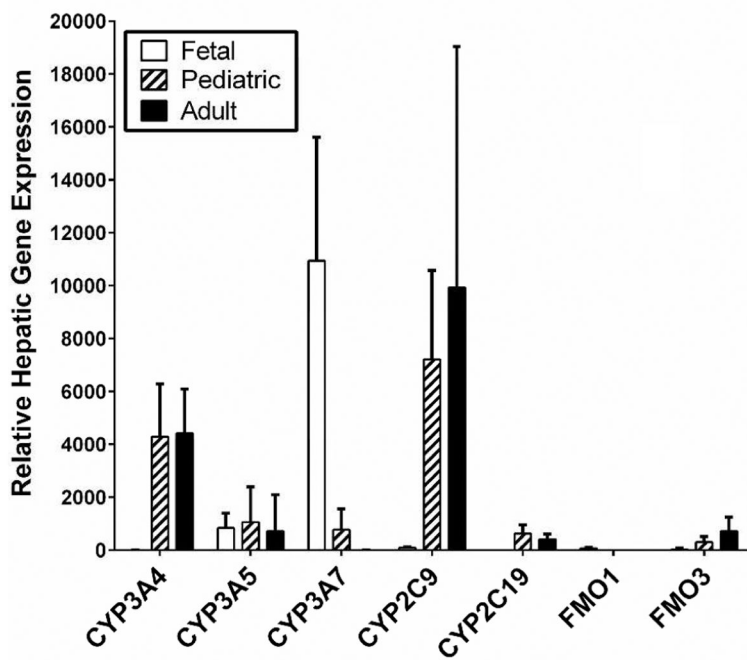
Figure 1. Expression of CYP and FMO Genes in Fetal, Pediatric and Adult Hepatic Tissues.
Gene expression was normalized to the lowest expressed enzyme, specifically adult FMO1, in order to show relative expression of all enzymes in all three populations. Fetal, pediatric, and adult samples are represented by blue, yellow, and green bars, respectively. Inserts depict relative expression of the CYP3A family [a], CYP2C family [b], and the FMO family [c].
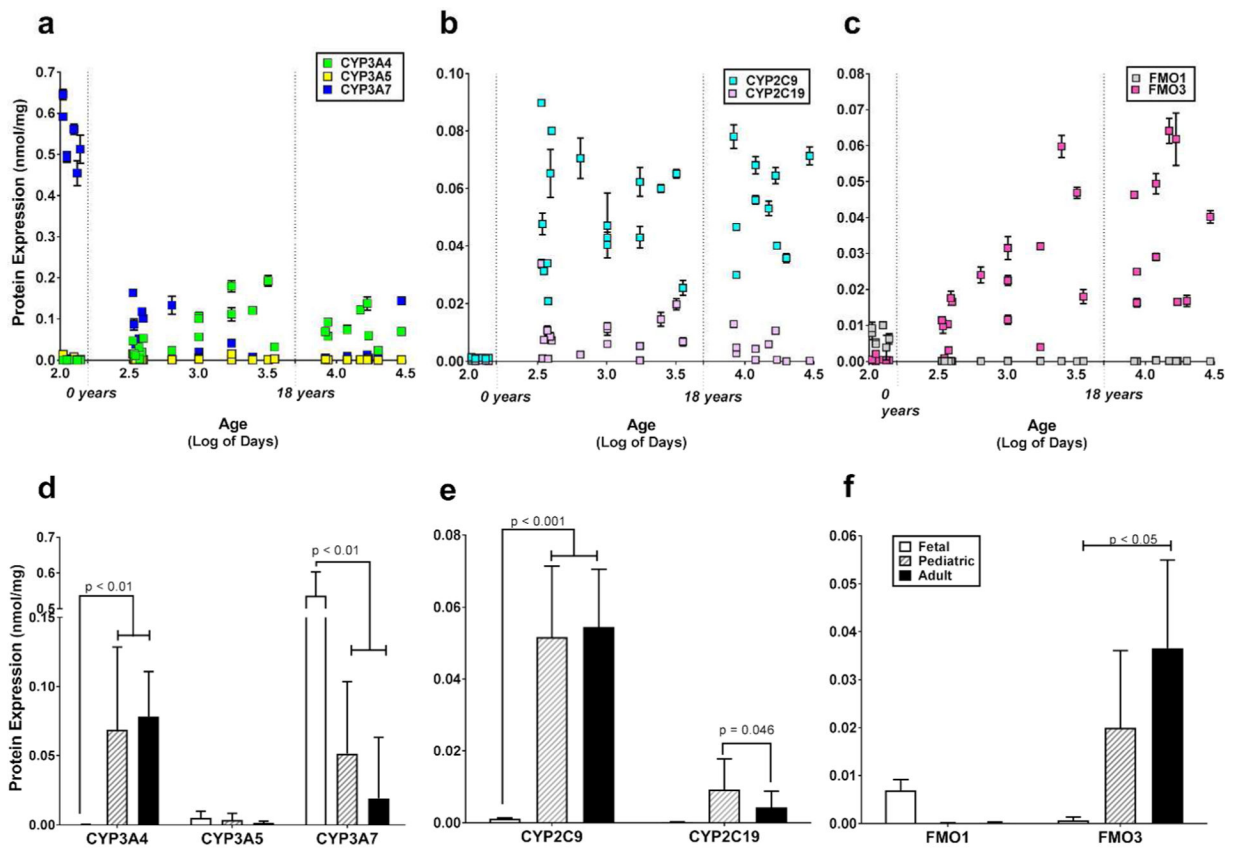
Figure 2. Expression of CYP3A, CYP2C, and FMO Proteins in Fetal, Pediatric, and Adult Hepatic Tissues.

Protein expression was measured in triplicate in fetal (n = 7), pediatric (n = 16), and adult (n = 10) HLM by LC-MRM-based targeted quantitative proteomic analysis. Panels depict protein expression of the CYP3A family [a], CYP2C family [b], and the FMO family [c]. For the CYP3A family, the green, yellow, and blue squares represent CYP3A4, CYP3A5, and CYP3A7 proteins, respectively. For the CYP2C family, the aqua and purple squares represent the CYP2C9 and CYP2C19 proteins, respectively. For the FMO family, the red, orange, and grey squares depict FMO1, FMO3, and FMO5 proteins, respectively. A comparison of CYP3A [d], CYP2C [e], and FMO [f] protein expression in the fetal (open bars), pediatric (grey hatched bars) and adult (solid bars) hepatic tissues is depicted as mean values ± standard deviation.
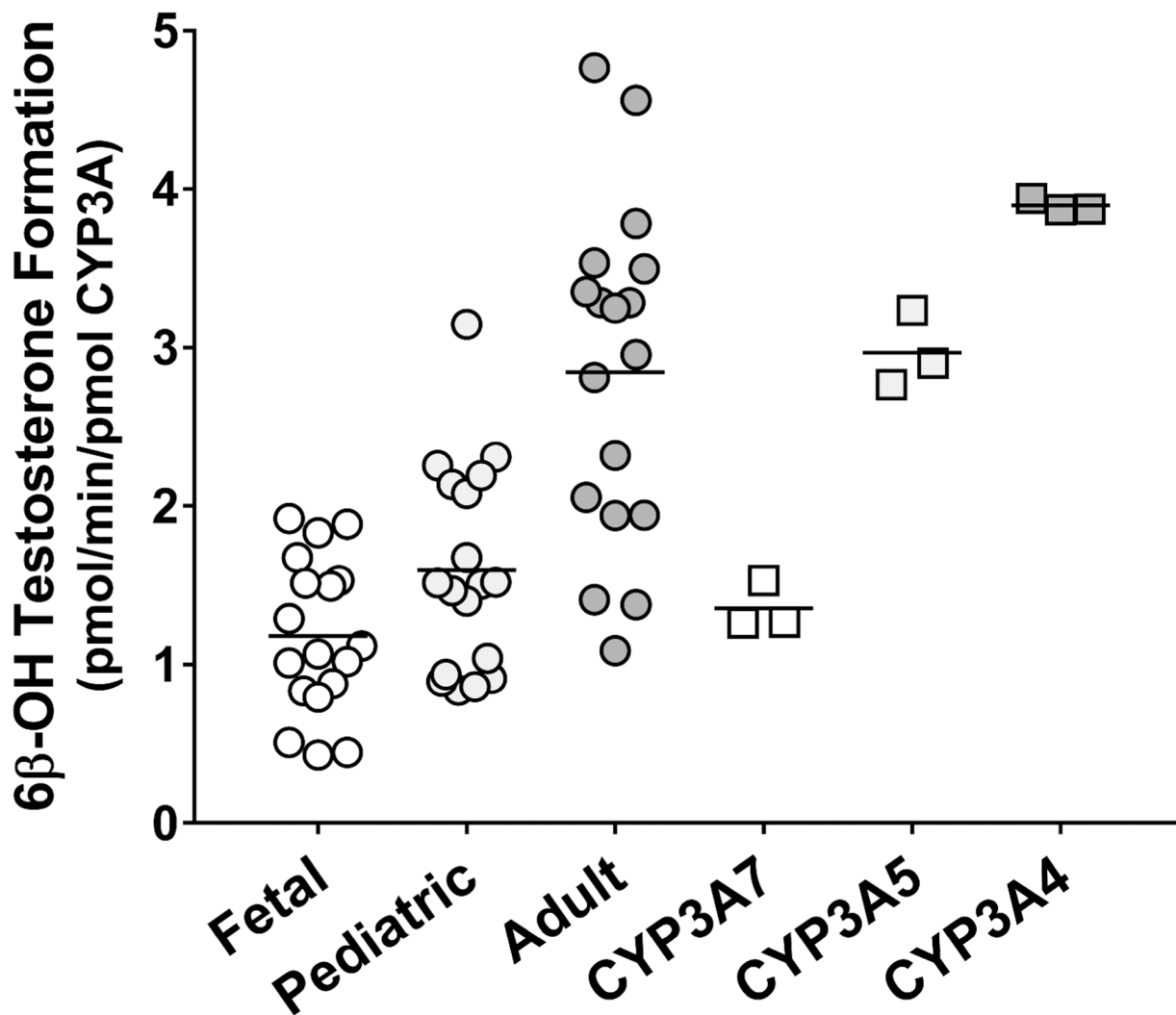
Figure 3. CYP3A Enzyme Activity in the Microsomes Prepared from Fetal, Pediatric, and Adult Human Hepatic Tissues and in Recombinant CYP3A Supersomes.
Fetal, pediatric, and adult HLM (n = 6) and recombinant CYP3A supersomes were incubated in triplicate with testosterone and the rate of formation of 6β-hydroxytestosterone was measured. The metabolite formation was normalized to the CYP3A expressed in each sample. The horizontal line in each group represents the mean value.
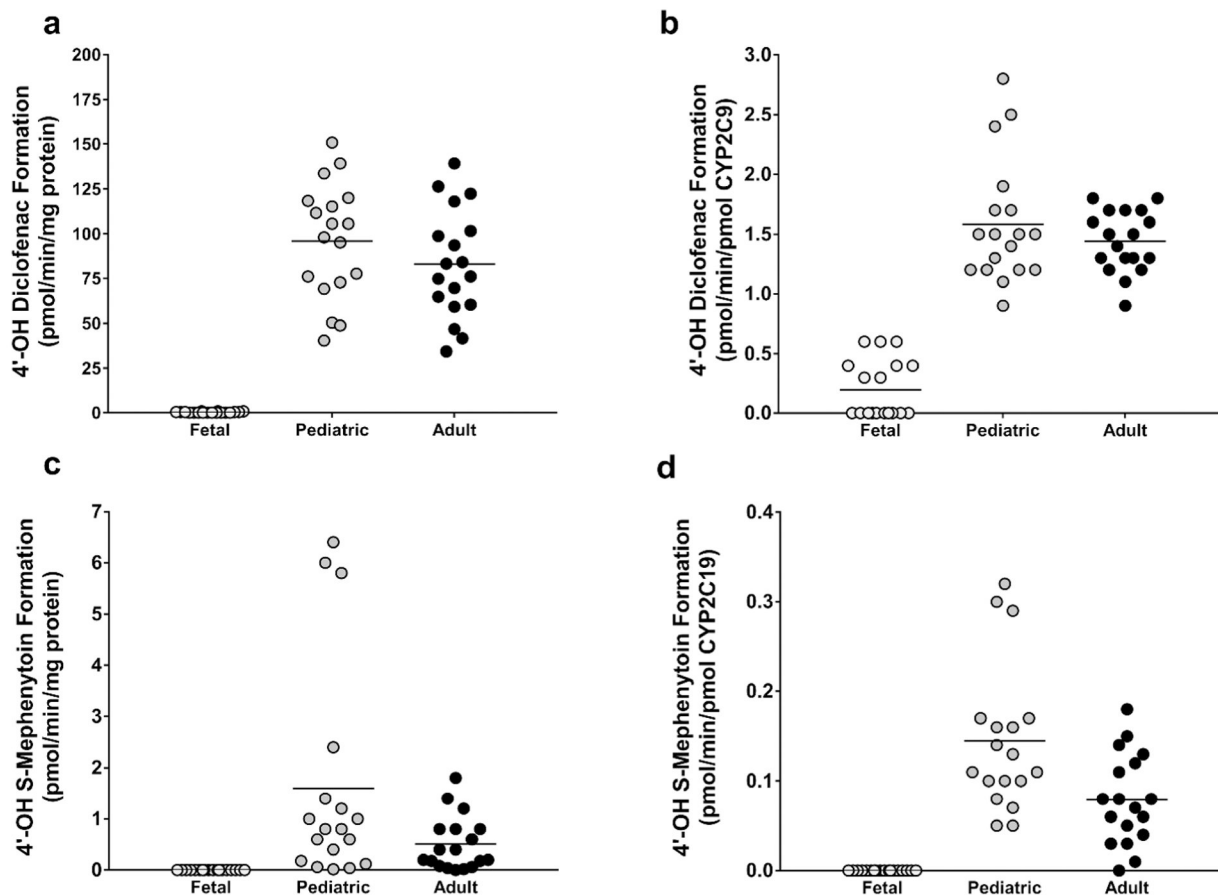
Figure 4. CYP2C Enzyme Activity in the Microsomes Prepared from Fetal, Pediatric, and Adult Human Hepatic Tissues.

Fetal, pediatric, and adult HLM (n = 6) were incubated in triplicate with diclofenac to measure CYP2C9 [a and b] and with S-mephenytoin to measure CYP2C19 [c and d] activity. Metabolite formation rates were normalized to the total microsomal protein [a, c], the CYP2C9 protein [b], and the CYP2C19 protein [d]. Horizontal lines represent the mean values in each panel.
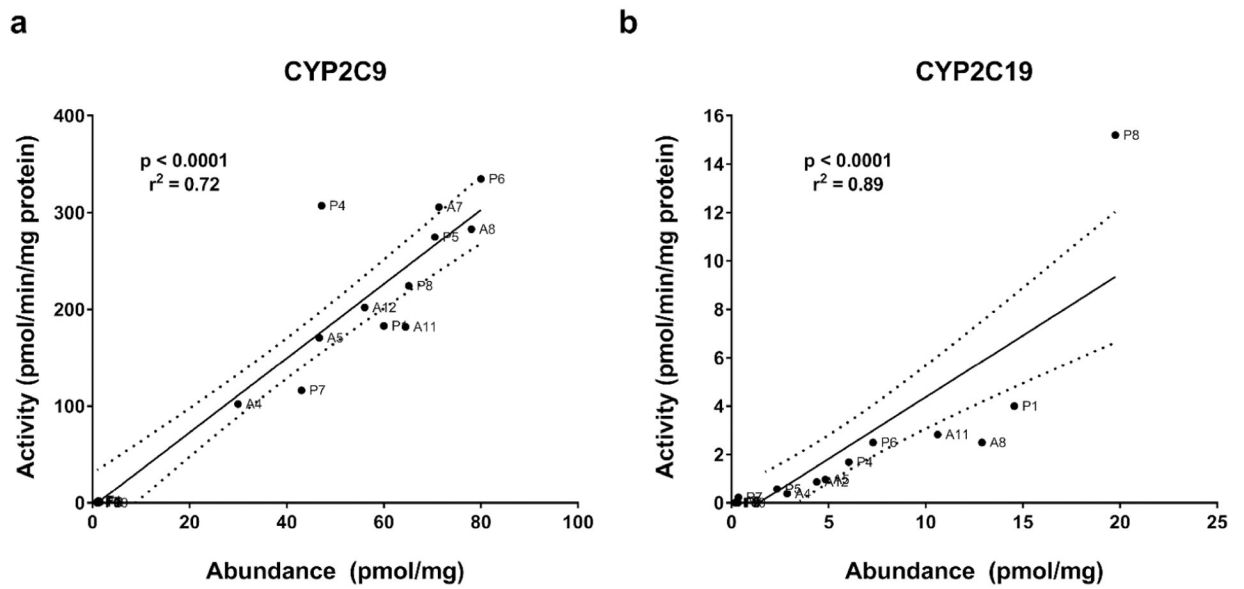
Figure 5. Correlation between CYP2C Protein Expression and Enzyme Activity.
Formation of 4'-OH diclofenac is correlated with the expression of CYP2C9 protein [a].
Formation of 4'-OH S-mephenytoin is correlated with the expression of CYP2C19 protein
[b]. The graphs depict the linear correlation (solid line) and the 95th confidence intervals
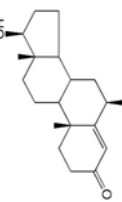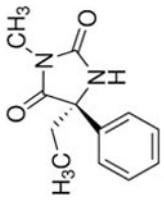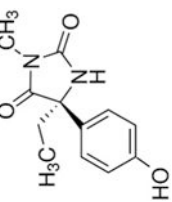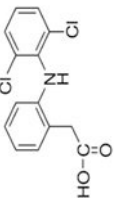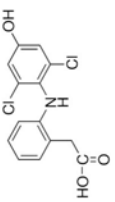(dotted lines).
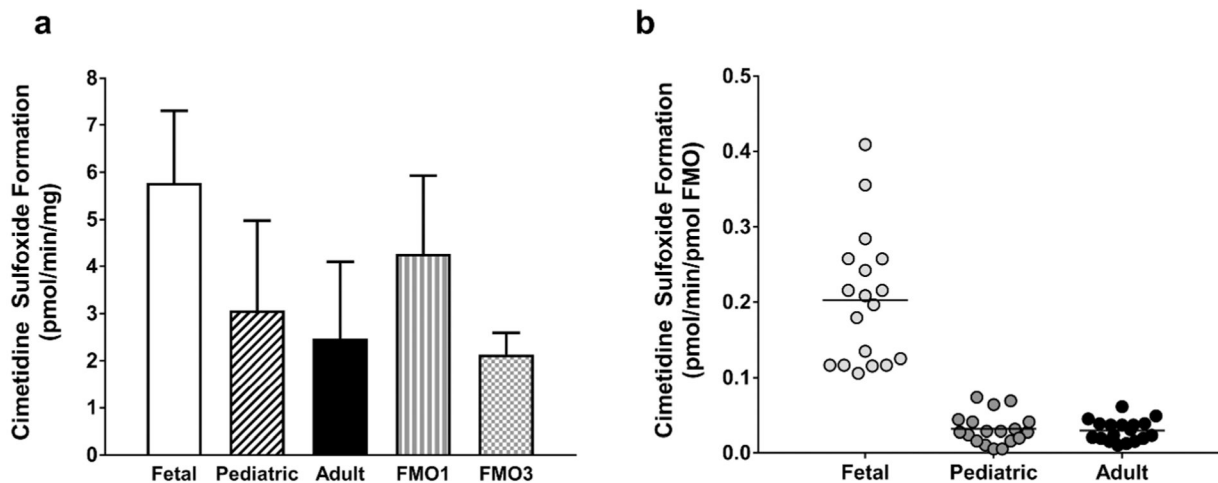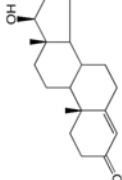
Figure 6. Enzyme Activity in the Microsomes Prepared from Fetal, Pediatric, and Adult Human Hepatic Tissues.
Fetal, pediatric, and adult HLM (n = 6) and recombinant FMO supersomes were incubated in triplicate with cimetidine to measure FMO activity. Metabolite formation rates were normalized to total microsomal protein [a] and the total FMO1 and FMO3 protein [b]. The bars represent values derived from six samples, and the error bars depict standard deviation [a]. Horizontal lines represent the mean values in each panel [b].

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Probe Substrates for Cytochrome P450 and FMO Enzymes.

Chemical structures of probe substrates and their major metabolites, as well as concentrations of the substrates used in phenotypic assays.

Enzyme	Probe	Parent Structure	[S]	Metabolite	Metabolite Structure
CYP3A	Testosterone		10 μM	6β-OH Testosterone	
CYP2C19	S-mephenytoin		10 μM	4′-OH S-mephenytoin	
CYP2C9	Diclofenac		10 μM	4′-OH Diclofenac	
FMO	Cimetidine		10 μM	Cimetidine Sulfoxide	