JPET Fast Forward. Published on November 21, 2003 as DOI:10.1124/jpet.103.060137

Developmental Expression of Human Hepatic CYP2C9 and CYP2C19

SEVASTI B. KOUKOURITAKI, JASON R. MANRO, SANDRA A. MARSH, JEFFREY C. STEVENS, ALLAN E. RETTIE, D. GAIL MCCARVER AND RONALD N. HINES

Department of Pediatrics, Birth Defects Research Center, Medical College of Wisconsin, Milwaukee, Wisconsin (S.B.K., D.G.M., R.N.H.); Pfizer, Pharmacokinetics, Dynamics, and Metabolism, Kalamazoo, Michigan (J.R.M., S.A.M., J.C.S.); and Department of Medicinal Chemistry, University of Washington, Seattle, Washington (A.E.R.)

Running Title: CYP2C9 and 2C19 Developmental Expression

Corresponding Author: Ronald N. Hines, Ph.D., Medical College of Wisconsin, Department of Pediatrics, Birth Defects Research Center, 8701 Watertown Plank Rd., Milwaukee WI 53226-4801, T: (414) 456-4322, F: (414) 456-6651, E-mail: rhines@mail.mcw.edu

Number of Text Pages: 29

Number of Tables: 4

Number of Figures: 6

Number of References: 39

Number of words in Abstract: 250

Number of words in Introduction: 604

Number of words in Discussion: 1455

Non-standard Abbreviations: constitutive androstane receptor, CAR; glucocorticoid receptor, GR; hepatocyte nuclear factor, HNF; pregnane X receptor, PXR

Recommended Section Assignment: Absorption, Distribution, Metabolism & Excretion

ABSTRACT

The CYP2C subfamily is responsible for metabolizing many important drugs and accounts for about 20% of the cytochrome P450 in adult liver. To determine developmental expression patterns, liver microsomal CYP2C9 and 2C19 were measured (N=237; ages=8 wks gestation-18 yrs) by western blotting and with diclofenac or mephenytoin, respectively, as probe substrates. CYP2C9 specific content and catalytic activity were consistent with expression at 1-2% of mature values (i.e., specific content=18.3 pmol/mg protein, N=79; specific activity=549.5 pmol/mg/min, N=72) during the first trimester with progressive increases during the second and third trimesters to levels approximately 30% of mature values. From birth to 5 mos, CYP2C9 protein values varied 35-fold and were significantly higher than that observed during the late fetal period with 51% of samples exhibiting values commensurate with mature levels. Less variable CYP2C9 protein and activity were observed between 5 mos and 18 yrs. CYP2C19 protein and catalytic activities that were 12-15% of mature values (i.e., specific content=14.6 pmol/mg, N=20; specific activity=18.5 pmol/mg/min, N=19) were observed as early as 8 wks of gestation and were similar throughout the prenatal period. CYP2C19 expression did not change at birth, increased linearly over the first 5 postnatal mos, and varied 21-fold from 5 mos to 10 yrs. Adult CYP2C19 protein and activity values were observed in samples older than 10 yrs. The ontogeny of CYP2C9 and 2C19 were dissimilar among both fetal and 0-5 mos postnatal samples, implying different developmental regulatory mechanisms.

INTRODUCTION

The cytochromes P450 (EC 1.14.14.1) comprise a superfamily of heme-thiolate enzymes, of which over 2,000 individual members are known representing species from all biological kingdoms (see Nelson database at http://drnelson.utmem.edu/CytochromeP450.html). In the human, 17 gene families exist, three of which, *CYP1*, *CYP2* and *CYP3*, encode enzymes involved in xenobiotic oxidative metabolism. Individual cytochrome P450 enzymes belonging to these families are characterized by diverse substrate specificity and significant differences in regulation, the latter resulting in temporal-, tissue-, and gender-dependent expression patterns (Gonzalez, 1990). Genetic variability among individual family members further contributes to significant inter-subject differences in metabolic capacity and pharmacological response (Rogers et al., 2002).

Studies on temporal-specific cytochrome P450 expression during ontogeny have been hampered by the low levels of enzyme generally present in the developing organism, overlapping substrate specificity that makes metabolic phenotyping difficult to interpret, and the paucity of suitable tissue samples for study. For individual cytochrome P450 enzymes, a limited number of studies suggest that expression during ontogeny is highly variable and among different cytochrome P450 enzymes within the same subfamily, often asynchronous (reviewed in Hines and McCarver, 2002). This temporal asynchrony raises important questions about the specific maturation of biotransformation pathways during ontogeny and an individual's ability to respond to xenobiotics during this time period.

The human *CYP2C* subfamily contains four highly homologous genes, *CYP2C8*, *2C9*, *2C18* and *2C19*, located in an approximate 500 kbp cluster on chromosome 10q24. The CYP2C subfamily accounts for about 18% of the total adult liver cytochrome P450 content (Shimada et al., 1994), the

major form being CYP2C9, followed by CYP2C19 and CYP2C8 (Edwards et al., 1998;Goldstein et al., 1994). Low levels of CYP2C mRNA and protein also have been detected in the small intestine and other extrahepatic tissues (Klose et al., 1999). Transcriptional regulation of both *CYP2C9* and *2C19* is known to involve the constitutive androstane receptor (CAR), pregnane X receptor (PXR), as well as the glucocorticoid receptor (GR) (Gerbal-Chaloin et al., 2002;Ferguson et al., 2002;Chen et al., 2003). Clinically-relevant genetic variability within the *CYP2C* locus also has been well documented (Goldstein, 2001).

Members of the CYP2C subfamily account for the metabolism of about 20% of clinically important drugs (Goldstein, 2001), including the anticoagulant, warfarin (Kaminsky and Zhang, 1997), the H_2 receptor antagonist, omeprazole,(Andersson et al., 1993), the antiepilectic agent phenytoin (Giancarlo et al., 2001), the antihyperglycemia sulfonylureas, tolbutamide (Wester et al., 2000) and glipizide (Kidd et al., 1999), and many non-steroidal anti-inflammatory agents (Leemann et al., 1993). The CYP2C enzymes also metabolize the endogenous compound, arachidonic acid, and as such, may play an important physiological role via the generation of bioactive eicosanoids (Capdevila et al., 1992).

Previous studies of human hepatic CYP2C developmental expression showed no detectable protein, or catalytic activity between 16 and 40 wks of gestation, although low levels of mRNA were observed in fetal tissue. Enzyme levels and parallel activity were greater in the first month after birth, reaching adult levels by 1 year of age, suggesting that CYP2C maturation depends only on postnatal age (Treluyer et al., 1997). However, these studies did not describe the developmental expression pattern of individual CYP2C isoforms. Given the differences in substrate specificity between the different CYP2C enzymes, such knowledge would improve the ability to predict age-related differences in the efficacy of therapeutic entities and the morbidity of numerous toxicants.

The objectives of the current study were to characterize the developmental expression pattern for human hepatic CYP2C9 and CYP2C19 and determine the overall interindividual variation in CYP2C9 and CYP2C19 expression as a function of age using the largest set of fetal and pediatric liver samples collected to date (Koukouritaki et al., 2002) .

MATERIALS AND METHODS

Materials. Polyclonal antibodies against recombinant purified CYP2C9 and CYP2C19 were elicited in New Zealand White rabbits as previously described (Sadeque et al., 1993). Horseradish peroxidase-conjugated goat anti-rabbit IgG, nitrocellulose membrane and enhanced chemiluminescence (ECL) western blotting kits were purchased from Amersham (Arlington Heights, IL, USA). Bench Mark pre-stained protein molecular weight standards were from Invitrogen (Carlsbad, CA, USA). The micro bicinchoninic acid protein assay reagent kit was from Pierce (Rockford, IL, USA). Diclofenac (sodium salt) was purchased from Sigma (St. Louis MO). (S)-Mephenytoin, 4'-hydroxymephenytoin, and 4'-hydroxydiclofenac were purchased from BD-Gentest (Woburn MA). All other reagents were obtained from commercial sources at the purest grade available.

Tissue samples. Frozen specimens of human liver were obtained from the Brain and Tissue Bank for Developmental Disorders, University of Baltimore and University of Miami (National Institute for Child Health and Human Development, NOI-HD-8-3283 and NOI-HD-8-3284, respectively) and the Central Laboratory for Human Embryology at the University of Washington (National Institute of Child Health and Human Development, HD-00836). Donor characteristics and exclusion criteria were as described previously (Koukouritaki et al., 2002). Tissue was stored at -80^{0}C until used for the preparation of microsomal suspensions. This study was approved by the Children's Hospital of Wisconsin and the Medical College of Wisconsin Institutional Review Boards. As a control for the metabolic assays, pooled adult human liver microsomes were obtained from Gentest Corporation (Woburn, MA).

Preparation of microsomes, electrophoresis and imunoblotting. Liver microsomes were prepared by differential centrifugation and analyzed for CYP2C9 and CYP2C19 specific content essentially as described previously (Koukouritaki et al., 2002). Briefly, 40 to 60 μg of microsomal protein from each liver sample were fractionated by sodium dodecyl sulfate polyacrylamide gel electrophoresis, using 10% resolving gels, along with 10, 25, 50, 100, 250 and 500 fmol of recombinant human CYP2C9 and CYP2C19 (Haining et al., 1996). Both the CYP2C9 and CYP2C19 primary antibodies were used at a final concentration of 5 μg/ml, diluted in Tris-buffered saline (25 mM Tris, pH 7.5, 150 mM sodium chloride) containing 0.5% nonfat dry milk. Horseradish-conjugated goat anti-rabbit IgG secondary antibody was diluted 1:5000 in Tris-buffered saline containing 0.5% non-fat dry milk. All incubations for both primary and secondary antibodies were performed for 90 min at room temperature. In all blots, bands corresponding to the protein of interest (CYP2C9 or CYP2C19) were identified by reference to the recombinant CYP2C9 and CYP2C19 and molecular weight standards. After determining the integrated optical densities of the digitized, immunoreactive bands (Kodak Digital Science ID Software), the CYP2C9 and CYP2C19 specific content of the patient samples was determined by linear regression based on a standard curve determined from the recombinant CYP2C9 and CYP2C19 included on each blot (GraphPad Instat version 3.00, GraphPad Software, Inc., San Diego, CA, U.S.A.). The limit of sensitivity of this assay was 10 fmol of CYP2C9 or CYP2C19.

Metabolic Assays. For both CYP2C9 and CYP2C19 activity determinations, incubations were performed in duplicate and under conditions previously determined to yield linear production of the respective marker metabolite (Stevens et al., 1997). For all incubations, 96-well format cluster tubes (Corning, Corning NY) were pre-cooled and kept on ice until initiating incubations. A mixture of cold substrate and buffer (155 μL total) was added at appropriate concentrations to each tube. A

25 µL aliquot of each microsomal sample (12.5 µg protein/ml final concentration) was transferred to individual tubes and after pre-incubating at 37°C for 3 min, reactions were initiated by the addition of 20 µL 10 mM NADPH. No NADPH was added to human liver microsome negative controls or standards. After 30 min, reactions were stopped by the addition of 50 µl cold acetonitrile. Samples were immediately placed on ice, capped and stored at -20°C. In preparation for analysis, plates were warmed to room temperature and centrifuged at 2,500 RPM. Supernatant fractions were aspirated, transferred to a clean 96-well plate and capped for analysis. The conversion of diclofenac (20 µM final assay concentration) to 4-hydroxydiclofenac was used to measure CYP2C9 metabolic activity in individual microsomal preparations. The product, 4-hydroxydiclofenac, was separated and quantified by HPLC using a previously described method (Fan et al., 2003). Samples areas were read from a linear regression of known standard amounts (range 40 to 5,000 pmol) using no weighting. Calculations to activity (pmol/min/mg) and averaging of replicates were performed in Excel (Microsoft, Seattle WA). Diclofenac 4-hydroxylase activity in pooled adult human liver microsomes (Gentest) was 1,340 ± 260 pmol/min/mg protein (N=42 determinations).

The conversion of (S)-mephenytoin to 4-hydroxymephenytoin was used to measure CYP2C19 metabolic activity in individual microsomal samples. The reaction product, 4-hydroxymephenytoin, was detected and quantified by HPLC/mass spectrometry as previously described (Fan et al., 2003). Samples areas were read from a linear regression of known standards (range 4.68 to 150.00 pmol) using 1/x weighting. Activities were calculated (pmol/min/mg) and replicates averaged in Excel (Microsoft). (S)-Mephenytoin 4-hydroxylase activity in pooled adult human liver microsomes (Gentest) was 44.0 ± 10.0 pmol/min/mg protein (N=41 determinations).

Data Analysis. Differences in CYP2C9 and 2C19 specific content among different age groups, as well as possible differences between the current study and specific activities previously reported were assessed using Kruskal-Wallis ANOVA with a Dunn's post hoc test. When raw data were not available, ANOVA with a Bonferonni correction for multiple comparisons was used to compare current data with previous reports. Differences between fetal and neonatal samples (0 to 30 days postnatal age) were tested using the Mann-Whitney U test. Differences between CYP2C9 and CYP2C19 specific content in individuals within age groups was examined using a paired Student's t test (Graphpad Instat, San Diego, CA). Linear regression was used to evaluate factors contributing to variation in CYP2C expression (SPSSPC, SPSS Inc., Chicago, IL). In all cases, $p < 0.05$ was accepted as indicating a significant difference.

JPET Fast Forward. Published on November 21, 2003 as DOI: 10.1124/jpet.103.060137
This article has not been copyedited and formatted. The final version may differ from this version.

RESULTS

Developmental expression patterns for CYP2C9 and CYP2C19. Western blotting with antibodies to CYP2C9 or CYP2C19 revealed immunoreactive bands with apparent molecular masses near 50 kD, corresponding to full-length CYP2C9 or CYP2C19, respectively. Figures 1A and 1B, depict representative western blots with human hepatic microsomes of different ages. Both CYP2C9 (Fig. 1A) and CYP2C19 (Fig. 1B) proteins were present at low levels in some fetal liver samples, but appeared to be much higher in postnatal liver samples. Coefficients of determination (r^2) for the standard curves ranged between 0.96 and 0.99 (median=0.98 for both CYP2C9 and CYP2C19, N=21) and coefficients of variation for independent experiments were 8% for CYP2C9 (N=5) and 6% for CYP2C19 (N=5). The less intense band with a mobility between CYP2C9 and CYP2C19 (some samples in Fig. 1B) is due to cross-reactivity of the CYP2C19 polyclonal antibody with CYP2C8. However, this signal did not interfere with the quantitation of either CYP2C9 or CYP2C19.

The fetal through early infancy developmental expression patterns for CYP2C9 and CYP2C19 are shown in Figs. 2A and 3A, respectively while the expression patterns for all postnatal samples (1 day to 18 years) are shown in Figs. 2B and 3B, respectively. Relative to mature values, low CYP2C9 protein levels were detected in many fetal samples between 8 and 24 wks of gestation. Progressively greater values were observed during the remaining gestational period such that some samples had specific contents of 10 to 12 pmol/mg, commensurate with values in the mature range (Fig. 2A). CYP2C19 also was detectable at 8 wks of gestation, but unlike CYP2C9, CYP2C19 specific content was similar among most gestational age samples (Fig. 3A). The CYP2C9 and CYP2C19 developmental expression patterns also were quite different after birth. CYP2C9 specific

content was significantly greater in neonatal samples (first 30 days after birth) than in fetal samples older than 24 wks of gestation (Mann-Whitney U, $p < 0.05$); however, variability was extensive over a constant range during the first 5 postnatal mos (Fig. 2A). In contrast, CYP2C19 protein levels were not significantly greater in the neonatal versus fetal samples (Mann Whitney U, $p > 0.05$), but rather, exhibited a somewhat linear increase over the first 5 mos postnatal age ($r^2 = 0.477$) (Fig. 3A). It appeared that several individual tissue samples within the perinatal grouping defined a subset with elevated CYP2C19 specific content (shown by open circles, Fig. 3A). Of interest, each of these samples were from individuals that were born prematurely (< 33 weeks). An examination of CYP2C9 specific content in all postnatal samples (1 day to 18 yr, N=166) (Fig. 2B) revealed that most samples from individuals 1 to 2 years postnatal age exhibited mature protein levels. Further, it was apparent that many individuals expressed CYP2C9 at levels equivalent to the maximum observed in the data set immediately after birth. In contrast, with the exception of one or two outliers, CYP2C19 maximum expression levels were not observed in individuals until after 5 months postnatal age. The delay in CYP2C19 expression also is apparent in 18 samples (33% of total) between 5 mos and 10 years that have values similar to those of fetal samples (Fig. 3B, hatched circles).

The developmental expression patterns for both CYP2C9 and CYP2C19 also were examined by measuring specific marker activities in individual microsomal preparations. Using CYP2C9-catalyzed diclofenac 4-hydroxylation (Tang et al., 1999), a pattern consistent with that observed by western blotting was observed (Table 1). Similarly, the conversion of mephenytoin to 4-hydroxymephenytoin was used as a metabolic marker for CYP2C19 (Goldstein et al., 1994) and again, a pattern consistent with protein measurements was observed (Table 2). However, individual CYP2C9 and CYP2C19 specific contents and specific activities did not correlate (data not shown).

Known coding region polymorphisms that result in reduced activity, but not reduced protein levels [for examples, see review by Goldstein (Goldstein, 2001)] may be a contributing factor to this lack of correlation. However, the lack of correlation was not a reflection of postmortem degradation and inactivation of the enzymes. The postmortem interval (time between death and freezing of liver samples) was from 1 to 41 hrs (N=214; median = 17 hrs). Neither specific content and postmortem interval or specific activity and postmortem interval were correlated (r^2=0.004 and 0.109, respectively). Further, excessive degradation was not seen on any of the western blots (see Fig. 1 as example).

The diclofenac 4-hydroxylase and (S)-mephenytoin 4'-hydroxylase activity in the oldest age brackets were compared to previously reported data. Diclofenac 4-hydroxylase specific activity in the oldest age bracket (> 5 mo to 18 yrs) was significantly lower than that reported by Yasar et al. (2001) or from the data available from BD Gentest (http://www.bdbiosciences.com) (Table 3). In contrast, the (S) mephenytoin 4-hydroxylase specific activity in the oldest age bracket (> 10 to 18 yrs) was significantly lower than data reported by Goldstein et al. (1994) and from the data obtained using an adult liver bank developed at Pfizer, but not from the specific activities reported by five other groups (Table 4).

Factors impacting CYP2C9 and CYP2C19 developmental expression. In adult liver samples, CYP2C9 has been shown to be present at higher levels than CYP2C19 (Edwards et al., 1998;Goldstein et al., 1994). This relationship holds for postnatal samples across development (Fig. 4B and C), but the reverse is true in fetal samples (Fig. 4A). Comparing all fetal samples (N = 71), CYP2C19 specific content was significantly greater than CYP2C9 specific content (paired t test, $p < 0.0001$). In contrast, comparing all samples in the early infancy period (0 to 5 months, N = 92) or those samples 5 months or older (N = 74), CYP2C9 specific content was significantly greater than

CYP2C19 specific content (paired t test, $p < 0.0001$ for both). No relationship existed between values of CYP2C9 and 2C19 during either the fetal or 0 to 5 mos postnatal age groups (linear regression, $r^2 < 0.05$, both), consistent with different factors regulating the ontogeny of both proteins. Finally, CYP2C9 and 2C19 content and activity in postnatal samples were each related to age (stepwise linear regression, $p < 0.05$ each), but were not related to gender, ethnicity or post mortem interval (stepwise linear regression, not significant).

Interindividual variation in CYP2C9 and CYP2C19 expression. To better summarize the different phases of CYP2C9 and CYP2C19 developmental expression, as well as quantify the interindividual variability in expression during different developmental stages, the data presented in Figs. 2 and 3 were divided into age groups to minimize differences within a group but maximize differences between groups. Results of this analysis are shown in Fig. 5 and 6. CYP2C9 developmental expression was divided into two prenatal and two postnatal phases (Fig. 5). In the mature samples (5 mos to 18 yrs), the 5th to 95th percentile values of CYP2C9 specific content ranged from 7.9 to 28.7 pmol/mg microsomal protein (4-fold). CYP2C9 specific content was observed in 84% of the samples from 8 to 24 wks of gestation, but was only 1 to 2% of mature values. CYP2C9 specific content was greater from 25 to 40 wks of gestation (Kruskal-Wallis, $p < 0.05$) with a median value 27% of that seen in the mature samples, but was variable (34-fold range between 5th and 95th percentiles). During the neonatal and early infancy period (up to 5 mos), 51% of the samples had CYP2C9 protein values that were commensurate with mature values, but again, expression was highly variable (35-fold range between 5th and 95th percentiles).

The 5th to 95th percentile values of CYP2C19 specific content in mature samples ranged from 8.1 to 20.8 pmol/mg microsomal protein (Fig. 6). CYP2C19 protein levels (5th and 95th percentiles) varied 10-fold throughout gestation with a median level that was 18% of the mature value. Postnatal

CYP2C19 expression appeared to be divided into three phases. From 5 mos to 10 yrs postnatal age CYP2C19 specific content was greater than earlier age brackets (Kruskal-Wallis, $p < 0.001$), but remained highly variable (21-fold range between 5^{th} and 95^{th} percentiles) and significantly lower than mature values (Kruskal-Wallis, $p < 0.05$).

DISCUSSION

This report is the first to document CYP2C9 and 2C19 protein and catalytic activity in the prenatal human liver, as well as describe the ontogeny of these two individual gene products. Measurements of both CYP2C9 specific content and catalytic activity are consistent with hepatic expression of this enzyme at 1 to 2% of mature values during the first trimester of fetal development with progressive increases in expression during the second and third trimesters to levels approximately 30% of mature values. Neonatal CYP2C9 specific content was significantly higher than that observed during the late fetal period, suggesting that increased postnatal CYP2C9 expression is linked to birth. However, expression levels were highly variable during the first 5 mos after birth, suggesting differences in developmental factors that may or may not have a genetic component. From 5 mos to 18 yrs, expression levels were greater than other age groups, approached adult values, and exhibited less variability.

CYP2C19 protein and catalytic activities that were 12 to 15% of mature values were observed in hepatic tissue samples as early as 8 wks of gestation. However, in contrast to the gestational increases in CYP2C9, CYP2C19 expression was similar among all gestational ages. The postnatal CYP2C19 expression pattern also was dissimilar to that of CYP2C9 with no significant change in expression between the fetal and immediate neonatal periods. A small subset (N=5) of neonatal samples exhibited greater than expected CYP2C19 specific content for this age group. Each of these samples was from a premature patient. We speculate that this elevated expression may have been due to the usual clinical practice of administering maternal glucocorticoids during preterm labor to enhance fetal lung maturation. Such a speculation is consistent with the reported existence of a glucocorticoid responsive element controlling *CYP2C19* expression (Chen et al., 2003).

CYP2C19 protein values increased somewhat linearly over the first 5 mos of postnatal age, suggesting birth is not sufficient for full postnatal expression. CYP2C19 expression was highly variable from 5 mos to 10 years, also consistent with differences in developmental factors that may or may not have a genetic component. A significant, but modest increase in CYP2C19 specific content was observed from 10 yrs through 18 yrs postnatal age that approached previously reported adult values.

Treluyer et al. (1997) previously reported that CYP2C was not detectable in human fetal liver between 16 and 40 weeks. Moreover CYP2C expression was reported to be extremely low in newborn liver, was greater in the first month after birth, and between 3 and 12 mos, was only 30% of adult levels, suggesting that maturation of CYP2C expression depended largely on postnatal factors. Possible differences between this report and the current study that might contribute to the inconsistent results include sample size within specific age brackets, sample quality, and assay sensitivity. A major limiting factor in comparing the two studies is that the report by Treyluyer et al. (1997) was limited to an aggregate CYP2C expression pattern. The current data are consistent with a more recent study by Nagata et al. (2003) in which expression profiling was used to examine differential gene expression between the human fetal and adult liver. CYP2C9 mRNA levels were detected in 17 to 37 week fetal liver samples (N=7) and were elevated 13-fold in postnatal samples (ages from 5 to 65 years, N = 14).

The median CYP2C19 specific content in the oldest age bracket of the current study, 14.9 pmol/mg microsomal protein (N = 20; median age = 15.2 yrs), was not significantly different than values available from BD Gentest (median = 25 pmol/mg, range = 6.0 to 49.0, N=5) (http://www.bdbiosciences.com/) or that predicted by extrapolating from previously reported CYP2C19.1 (S)-mephenytoin 4-hydroxylase turnover [6.17 ± 0.24 nmol/min/nmol (Goldstein et al.,

1994)] and the adult liver microsomal (S)-mephenytoin 4-hydroxylase specific activities reported by Wrighton et al. (1993) (Kruskal-Wallis ANOVA, $p > 0.05$). Consistent with this observation, the (S)-mephenytoin 4-hydroxylase specific activities of the pediatric samples in the oldest age bracket of the current study (> 10 to 18 yrs) was not significantly different than most of the previously reported values (Table 4) and is consistent with the activity determined in a sample of pooled adult liver microsomes obtained from BD Gentest (44 ± 10 pmol/min/mg).

In contrast to what was observed with CYP2C19, the observed CYP2C9 specific content in the oldest age bracket (>5 mos through 18 yrs; N = 79; median = 5.0 yrs) of 18.0 ± 6.0 pmol/mg microsomal protein is significantly less than the total CYP2C specific content of 60 ± 27 pmol/mg protein (N=60; median age=54.5 yrs) reported by Shimada et al. (1994) (ANOVA, $p < 0.001$). However, the maximal CYP2C9 specific content determined in the current study may not reflect true adult values, i.e., an increase in mean activity would occur with age. Such a possibility is supported by the study of Treluyer et al. (1997), who reported a CYP2C specific content in liver samples from 3 to 12 month old individuals that was one third the CYP2C specific content observed in adult samples. A further increase in CYP2C9 expression with age also is supported by the significantly lower diclofenac 4-hydroxylase activities in the oldest age bracket of the current study (> 5mos to 18 yrs) compared to the data available from BD Gentest and the data reported by Yasar et al. (2001) (Table 3). Significantly lower activity in these same pediatric samples also is noted when compared to the mean activity of 2,300 ± 100 pmol/min/mg microsomal protein reported by Tang et al. (1999) for 10 pooled adult liver microsomal samples (age not specified), as well as the value measured for pooled adult microsomes (BD Gentest) in the current study, 1,340 ± 260 pmol/min/mg microsomal protein.

The reported dominant expression of *CYP2C9* over *CYP2C19* in adult liver (Goldstein et al., 1994) is in agreement with the postnatal data presented in the current study, although the degree of difference is not as striking. However, our data is consistent with an expected increase in mean CYP2C9, but not CYP2C19 expression with age (see discussion above). Such a change would result in a further increase in the CYP2C9/CYP2C19 ratio depicted in Fig. 4C. Of interest, the inverse relationship is observed in prenatal samples, i.e., CYP2C19 represents the dominant enzyme in most samples. Multiple factors have been implicated in regulating these two genes. Both *CYP2C9* and *CYP2C19* are known to be regulated by PXR, CAR, and GR (Gerbal-Chaloin et al., 2002;Ferguson et al., 2002;Chen et al., 2003). However, only the proximal *CYP2C19* CAR binding site was found to be active in contrast to both the distal and proximal CAR binding sites being functional in *CYP2C9*. This observation has been offered as a probable cause for lower constitutive CYP2C19 expression in adult human liver (Chen et al., 2003). Recent studies also have shown that CAR expression is markedly lower in the fetal and neonatal versus adult liver (Wei et al., 2002;Huang et al., 2003). Thus, it would appear that CAR has a minimal role in regulating fetal and neonatal hepatic *CYP2C* expression. Members of the hepatocyte nuclear factor (HNF) family of transcription factors that regulate hepatic-specific gene expression during development (Cereghini, 1996) also have been shown to be important for regulating several *CYP2C* subfamily members (Ibeanu and Goldstein, 1995) and as such, may have a role in regulating both fetal *CYP2C9* and *CYP2C19* expression. In the mouse, it recently has been reported that HNF4α also can regulate the induction of genes encoding xenobiotic metabolizing enzymes by controlling hepatic PXR transcription and that the up-regulation of PXR during fetal development is due to an increase in HNF4α expression (Kamiya et al., 2003). If a similar role is demonstrated in the human, HNF4α may directly regulate *CYP2C9* and *CYP2C19* developmental expression, as well as indirectly regulate these two genes

through its action on PXR. However, such regulation would not explain the differences in relative CYP2C9 and CYP2C19 expression between the fetal and postnatal periods. HNF4α also has been shown to be an important modulator of PXR- and CAR-dependent *CYP3A4* induction in the liver (Tirona et al., 2003) and as such, may be playing a similar role for *CYP2C* expression.

The maturation of organ systems during fetal, neonatal and childhood periods exerts a profound effect on drug disposition. Accordingly, effective and safe drug therapy in neonates, infants and children requires a thorough understanding of the dynamic ontogeny of drug absorption, distribution, metabolism and excretion. The current study demonstrates that CYP2C9 and CYP2C19 expression patterns were highly dissimilar among fetal, neonatal and early infancy samples, consistent with different mechanisms regulating the developmental expression of these two genes. An important contribution of developmental factors also is supported by the greater interindividual variation in CYP2C9 and CYP2C19 expression observed during the neonatal and early infancy periods.

References

Andersson T, Miners JO, Veronese ME, Tassaneeyakul W, Meyer UA and Birkett DJ (1993) Identification of human liver cytochrome P450 isoforms mediating omeprazole metabolism. *Br J Clin Pharmacol* **36**:521-530.

Capdevila JH, Falck JR and Estabrook RW (1992) Cytochrome P450 and the arachidonate cascade. *FASEB J* **6**:731-736.

Cereghini S (1996) Liver-enriched transcription factors and hepatocyte differentiation. *FASEB J* **10** :267-282.

Chen Y, Ferguson SS, Negishi M and Goldstein JA (2003) Identification of constitutive androstane receptor and glucocorticoid receptor binding sites in the *CYP2C19* promoter. *Mol Pharmacol* **64**:316-324.

Edwards RJ, Adams DA, Watts PS, Davies DS and Boobis AR (1998) Development of a comprehensive panel of antibodies against the major xenobiotic metabolising forms of cytochrome P450 in humans. *Biochem Pharmacol* **56**:377-387.

Fan PW, Gu C, Marsh SA and Stevens JC (2003) Mechanism-based inactivation of cytochrome P450 2B6 by a novel terminal acetylene inhibitor. *Drug Metab Dispos* **31**:28-36.

Ferguson SS, LeCluyse EL, Negishi M and Goldstein JA (2002) Regulation of human *CYP2C9* by the constitutive androstane receptor: Discovery of a new distal binding site. *Mol Pharmacol* **62**:737-746.

Gerbal-Chaloin S, Daujat M, Pascussi J-M, Pichard-Garcia L, Vilarem M-J and Maurel P (2002) Transcriptional regulation of CYP2C9 gene: Role of glucocorticoid receptor and constitutive adrostane receptor. *J Biol Chem* **277**:209-217.

Giancarlo GM, Venkatakrishnan K, Granda BW, Von Moltke LL and Greenblatt DJ (2001) Relative contribution of CYP2C9 and 2C19 to phenytoin 4-hydroxylation in vitro: Inhibition by sulfaphenazole, omeprazole, and ticlopidine. *Eur J Clin Pharmacol* **57**:31-36.

Goldstein JA (2001) Clinical relevance of genetic polymorphisms in the human CYP2C subfamily. *Br J Clin Pharmacol* **52**:349-355.

Goldstein JA, Faletto MB, Romkes-Sparks M, Sullivan T, Kitareewan S, Raucy JL, Lasker JM and Ghanayem BI (1994) Evidence that CYP2C19 is the major (*S*)-mephenytoin 4'- hydroxylase in humans. *Biochemistry* **33**:1743-1752.

Gonzalez FJ (1990) Molecular genetics of the P-450 superfamily. *Pharmac Ther* **45**:1-38.

Haining RL, Hunter AP, Veronese ME, Trager WF and Rettie AE (1996) Allelic variants of human cytochrome P450 2C9: Baculovirus- mediated expression, purification, structural characterization, substrate stereoselectivity, and prochiral selectivity of the wild-type and I359L mutant forms. *Arch Biochem Biophys* **333**:447-458.

Hines RN and McCarver DG (2002) The ontogeny of human drug metabolizing enzymes: Phase I oxidative enzymes. *J Pharmacol Exp Ther* **300**:355-360.

Huang W, Zhang J, Chua SS, Qatanani M, Han Y, Granata R and Moore DD (2003) Induction of bilirubin clearance by the constitutive adrostane receptor (CAR). *Proc Natl Acad Sci USA* **100**:4156-4161.

Ibeanu GC and Goldstein JA (1995) Transcriptional regulation of human CYP2C genes: Functional comparison of CYP2C9 and CYP2C18 promoter regions. *Biochemistry* **34**:8028-8036.

Kaminsky LS and Zhang ZY (1997) Human P450 metabolism of warfarin. *Pharmacol Ther* **73**:67-74.

Kamiya A, Inoue Y and Gonzalez FJ (2003) Role of the hepatocye nuclear factor 4 in control of the pregnane X receptor during fetal liver development. *Hepatology* **37**:1375-1384.

Kidd RS, Straughn AB, Meyer MC, Blaisdell J, Goldstein JA and Dalton JT (1999) Pharmacokinetics of chlorpheniramine, phenytoin, glipizide and nifedipine in an individual homozygous for the CYP2C9*3 allele. *Pharmacogenetics* **9**:71-90.

Klose TS, Blaisdell JA and Goldstein JA (1999) Gene structure of CYP2C8 and extrahepatic distribution of the human CYP2Cs. *J Biochem Mol Toxicol* **13**:289-295.

Koukouritaki SB, Simpson P, Yeung CK, Rettie AE and Hines RN (2002) Human hepatic flavin-containing monooxygenase 1 (*FMO1*) and 3 (*FMO3*) developmental expression. *Pediatric Res* **51**:236-243.

Leemann TD, Transon C, Bonnabry P and Dayer P (1993) A major role for cytochrome P450TB (CYP2C subfamily) in the actions of non-steroidal antiinflammatory drugs. *Drugs Exp Cllin Res* **19**:189-195.

Nagata T, Takahashi Y, Ishii Y, Asai S, Sugahara M, Nishida Y, Murata A, Chin M, Schichino H, Koshinaga T, Fukuzawa M and Mugishima H (2003) Profiling of genes differentially expressed between fetal liver and postnatal liver using high-density oligonucleotide DNA array. *Int J Mol Med* **11**:713-721.

Rogers JF, Nafziger AN and Bertino JS, Jr. (2002) Pharmacogenetics affects dosing, efficacy, and toxicity of cytochrome P450-metabolized drugs. *American Journal of Medicine* **113**:746-750.

Sadeque AJM, Thummel KE and Rettie AE (1993) Purification of macaque liver flavin-containing monooxygenase: A form of the enzyme related immunochemically to an isozyme expressed selectively in adult human liver. *Biochim Biophys Acta Protein Struct Mol Enzymol* **1162**:127-134.

Shimada T, Yamazaki H, Mimura M, Inui Y and Guengerich FP (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.

Stevens JC, White RB, Hsu SH and Martinet M (1997) Human liver CYP2B6-catalyzed hydroxylation of RP 73401. *J Pharmacol Exp Ther* **282**:1389-1395.

Tang W, Stearns RA, Wang RW, Chiu SH and Baillie TA (1999) Roles of human hepatic cytochrome P450s 2C9 and 3A4 in the metabolic activation of diclofenac. *Chem Res Toxicol* **12**:192-199.

Tirona RG, Lee W, Leake BF, Lan LB, Cline CB, Lamba V, Parviz F, Duncan SA, Inoue Y, Gonzalez FJ, Schuetz EG and Kim RB (2003) The orphan nuclear receptor HNF4alpha determines PXR- and CAR-mediated xenobiotic induction of CYP3A4. *Nat Med* **9**:220-224.

Treluyer J-M, Gueret G, Cheron G, Sonnier M and Cresteil T (1997) Developmental expression of CYP2C and CYP2C-dependent activities in the human liver: In vivo/in vitro correlation and inducibility. *Pharmacogenetics* **7**:441-452.

Wei P, Zhang J, Dowhan DH, Han Y and Moore DD (2002) Specific and overlapping functions of the nuclear hormone receptors CAR and PXR in xenobiotic response. *Pharmacogenomics J* **2**:117-126.

Wester MR, Lasker JM, Johnson EF and Raucy JL (2000) CYP2C19 participates in tolbutamide hydroxylation by human liver microsomes. *Drug Metab Dispos* **28**:354-359.

Wrighton SA, Stevens JC, Becker GW and Vandenbranden M (1993) Isolation and characterization of human liver cytochrome P450 2C19: Correlation between 2C19 and *S*-mephenytoin 4'-hydroxylation. *Arch Biochem Biophys* **306**:240-245.

Yasar Ü, Eliasson E, Forslund-Bergengren C, Tybring G, Gadd M, Sjöqvist F and Dahl M-L (2001) The role of *CYP2C9* genotype in the metabolism of diclofenac in vivo and in vitro. *Eur J Clin Pharmacol* **57**:729-735.

Footnotes

These studies were supported in part by funds from the Children's Hospital Foundation of Wisconsin (R.N.H. and D.G.M.) and National Institutes of Health grant GM32165 (A.E.R.).

Address correspondence to: Ronald N. Hines, Medical College of Wisconsin, Department of Pediatrics, Birth Defects Research Center, 8701 Watertown Plank Rd., Milwaukee WI 53226-4801, U.S.A.

Figure Legends

Fig. 1. Western blot analysis of CYP2C9 and 2C19 expression in human liver microsomes. Forty to sixty micrograms of microsomal protein were fractionated by SDS-PAGE, electrophoretically transferred to nitrocellulose membranes. The blots were probed with polyclonal antibodies raised against human CYP2C9 (A) or CYP2C19 (B) followed by secondary antibody and detection using enhanced chemiluminescence with a 10 min exposure. Lanes 1-5: 25, 50, 100, 250 and 500 fmol of recombinant human CYP2C9 and CYP2C19, respectively; lanes 6-10: five randomly selected prenatal liver samples representing various ages with the lower inset panel showing a 25 min exposure of the same blot; lanes 11-21: ten randomly selected postnatal liver samples representing various ages. The closed arrows indicate the mobility of the recombinant CYP2C9 (A) or CYP2C19 (B) protein, respectively while the open arrows indicate the corresponding antigen detected in the microsomal protein samples.

Fig. 2. Developmental expression pattern of CYP2C9. (A) A scatter plot analysis of CYP2C9 specific content as a function of gestational age (weeks) is depicted along with the transition at birth, the neonatal and early infancy period. (B) A scatter plot analysis of CYP2C9 specific content as a function of postnatal age (years) is depicted. Specific content was determined based on the density of the respective immunoreactive bands and a linear regression analysis based on the standard curve determined from the recombinant protein included on each blot (see Fig. 1 as an example).

Fig. 3. Developmental expression pattern of CYP2C19. (A) A scatter plot analysis of CYP2C19 specific content as a function of gestational age (weeks) is depicted, along with the transition at birth, the neonatal and early infancy period. The coefficient of determination (r^2) was calculated using Graphpad Instat (San Diego, CA) after excluding 9 outliers (open and hatched circles) based on a residual analysis. (B) A scatter plot analysis of CYP2C19 specific content as a function of postnatal age (years) is depicted. Samples from individuals greater than 5 mos postnatal age with specific content commensurate with fetal samples are depicted by the hatched circles. Specific content was determined based on the density of the respective immunoreactive bands and a linear regression analysis based on the standard curve determined from the recombinant protein included on each blot (see Fig. 1 as an example).

Fig. 4. Individual CYP2C9 and CYP2C19 specific content. A scatter plot correlating CYP2C9 and CYP2C19 specific contents in individual liver samples. (A) Fetal samples (8 to 40 wks of gestation); (B) Neonatal and early infancy samples (birth to 5 mos postnatal age); and (C) Samples greater than 5 mos to 18 years. The line of identity is shown for each age group. The significance of the observed difference was determined using a paired Student's t test.

Fig. 5. Determination of interindividual variability within defined age brackets during CYP2C9 developmental expression. A box and whisker plot analysis of the complete data set of CYP2C9 specific content is depicted. The boxes represent the 25^{th} to 75^{th} percentiles and the horizontal bar the median of the data. The vertical bars represent 5^{th} to 95^{th} percentiles. Fetal samples are shown by the shaded boxes while postnatal samples are shown by the open boxes. Outliers (open circles) were

defined as having specific contents outside 1.5 times the 25th or 75th percentiles. The sample size within each bracket (N), age ranges, and mean CYP2C9 specific content (± standard deviation, pmol/mg microsomal protein) also are shown. The calculations of mean ± standard deviations excluded outliers. Eighteen percent of the samples had non-detectable CYP2C9 in the earliest age bracket, but none in the remaining groups. Significant differences between groups as determined by Kruskal-Wallis non-parametric analysis and Dunn's multiple comparisons test are shown by asterisks (* $p < 0.05$; ** $p < 0.01$).

Fig. 6. Determination of interindividual variability within defined age brackets during CYP2C19 developmental expression. A box and whisker plot analysis of the complete data set of CYP2C19 specific content is depicted. The boxes represent the 25th to 75th percentile and the horizontal bar the median of the data. The vertical bars represent 5th to 95th percentile of the data. Fetal samples are shown by the shaded box while postnatal samples are shown by the open boxes. Outliers (open circles) were defined as having specific contents outside 1.5 times the 25th or 75th percentiles. The sample size within each bracket (N), age ranges, mean CYP2C19 specific content (± standard deviation, pmol/mg microsomal protein). The calculations of mean ± standard deviations excluded outliers. Single samples in each of the first two age brackets had no detectable CYP2C19, but none in the remaining age groups. Significant differences between groups as determined by Kruskal-Wallis non-parametric analysis and Dunn's multiple comparisons test are shown by asterisks (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

Table 1. Ontogeny of CYP2C9 Diclofenac 4-Hydroxylase Metabolic Activity

Age Bracket	Sample Size	%ND	Median Specific Activity[1] (pmol/min/mg)	Specific Activity Range[1] (pmol/min/mg)
Fetal				
8-40 wks	48	60	143	19 - 254
25-40 wks	16	38	195	37 - 735
Postnatal				
0 to 5 mo	88	16	899	53 - 4,600
> 5 mo to 18 yr	71	14	628	60 - 1,996

[1] Values for those samples that exhibited detectable activity

Table 2. Ontogeny of CYP2C19 (S)-Mephenytoin 4-Hydroxylase Metabolic Activity

Age Bracket	Sample Size	%ND	Median Specific Activity[1] (pmol/min/mg)	Specific Activity Range[1] (pmol/min/mg)
Fetal				
8-40 weeks	64	55	1.3	0.1 - 345.5
Postnatal				
0 to 5 mo	88	30	6.1	0.1 - 1,556.5
> 5 mo to 10 yr	52	12	22.4	0.1 - 656.5
> 10 yr to 18 yr	19	0	18.5	0.4 - 101.7

[1] Values for those samples that exhibited detectable activity

Table 3. Comparisons of CYP2C9 Diclofenac 4-Hydroxylase Metabolic Activity

Data Source	Sample Size	Median Age (yrs)	Median Specific Activity[1] (pmol/min/mg)	Specific Activity Range[1] (pmol/min/mg)
Current Study >5 mo to 18 yr	61	4.9	628	60 - 1,996
Yasar et al. (2001)	11	NA	1,555	217 - 4,781
BD Gentest[2]	12	47	2,515	1,020 - 3,970

[1] Values for those samples that exhibited detectable activity

[2] http://www.bdbiosciences.com/discovery_labware/gentest/products/

Current Study vs. Yasar et al. (2001), $P < 0.05$

Current Study vs. BD Gentest, $P < 0.001$

Table 4. Comparisons of CYP2C19 (S) Mephenytoin 4-Hydroxylase Metabolic Activity

Data Source	Sample Size	Median Age (yrs)	Median Specific Activity[1] (pmol/min/mg)	Specific Activity Range[1] (pmol/min/mg)
Current Study >10 to 18 yr	19	15.2	18.5	0.4 - 101.7
Chiba et al. (1993)	14	NA	55.5	15.0 - 173.0
Goldstein et al. (1994)[2]	15	NA	145.0	12.0 - 388.0
Pearce et al. (1996)	9	51	38.4	6.9 - 86.5
Wrighton et al. (1993)	13	29.5	66.0	2.0 - 310.0
Heyn et al. (1996)	14	NA	39.5	9.0 - 319.0
BD Gentest[2, 3]	10	46.0	26.5	4.0 - 320.0

[1] Values for those samples that exhibited detectable activity

[2] Excluded single outlier values with specific activity > 600 pmol/min/mg

[3] http://www.bdbiosciences.com

Current Study vs. Goldstein et al. (1994), $P < 0.001$(Kruskal-Wallis); Differences among all other groups were not significant

JPET Fast Forward. Published on November 21, 2003 as DOI: 10.1124/jpet.103.060137
This article has not been copyedited and formatted. The final version may differ from this version.

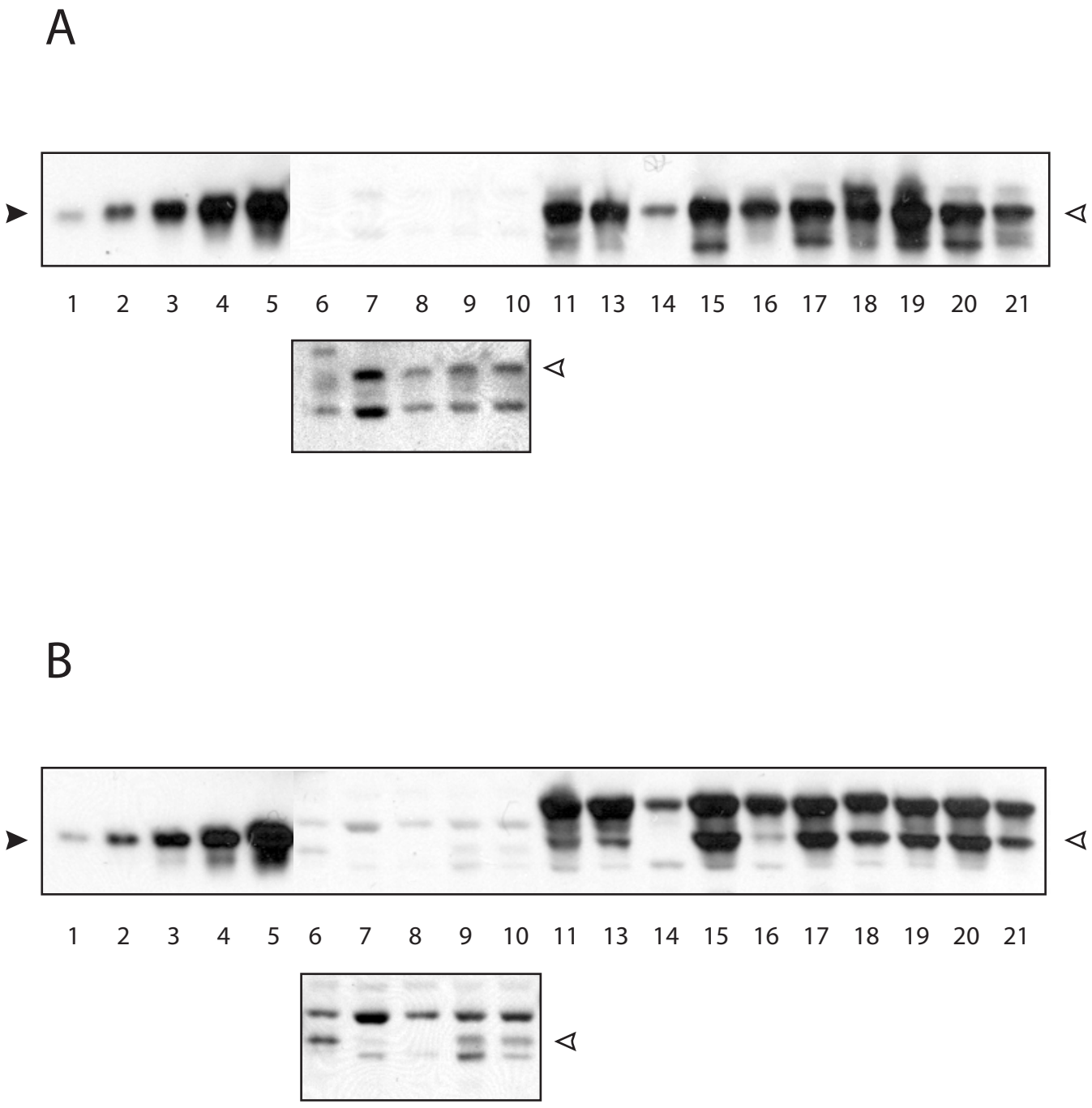

Fig 1, Koukouritaki et al.

JPET Fast Forward. Published on November 21, 2003 as DOI: 10.1124/jpet.103.060137
This article has not been copyedited and formatted. The final version may differ from this version.

A

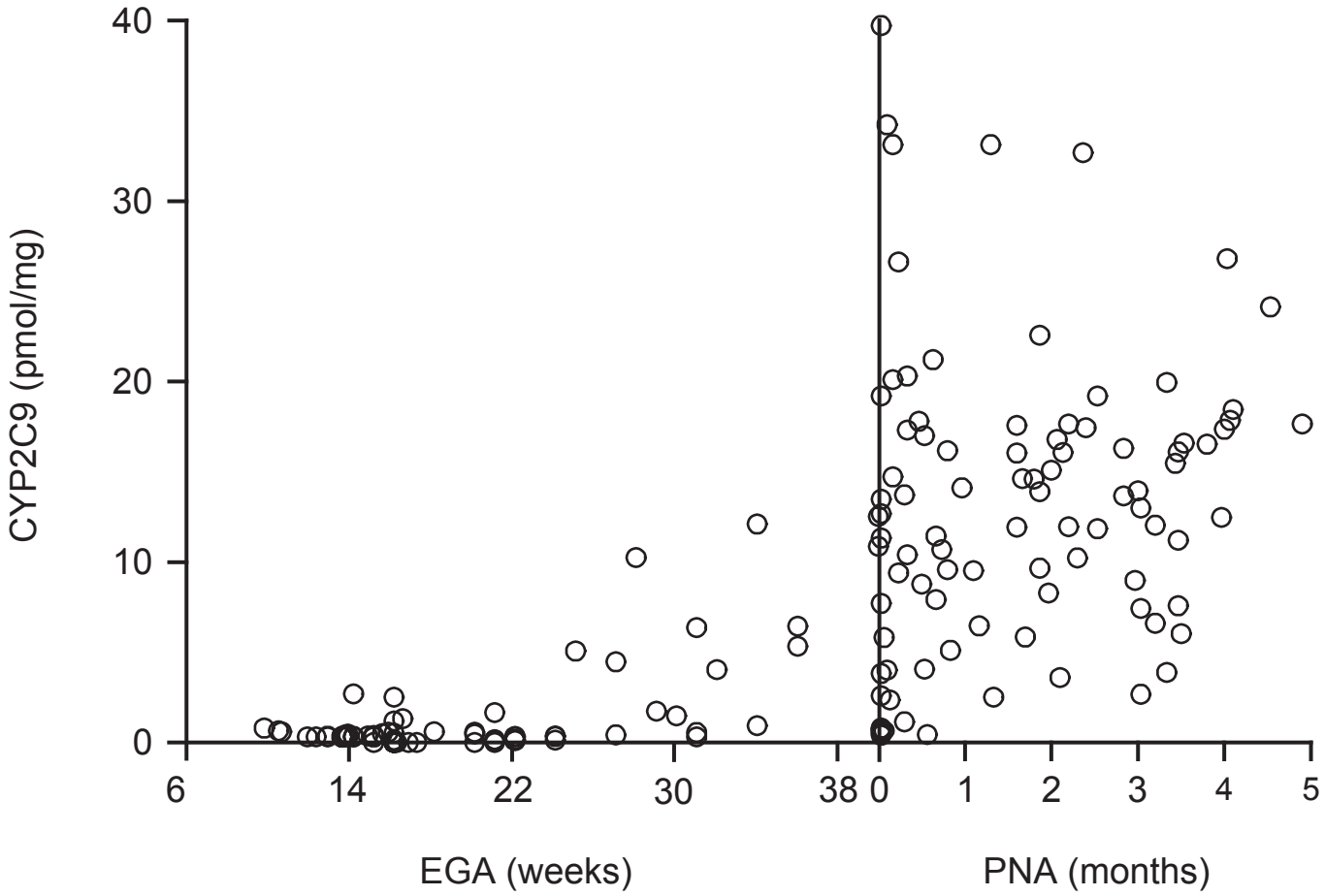

Fig 2A, Koukouritaki et al.

JPET Fast Forward. Published on November 21, 2003 as DOI: 10.1124/jpet.103.060137
This article has not been copyedited and formatted. The final version may differ from this version.

B

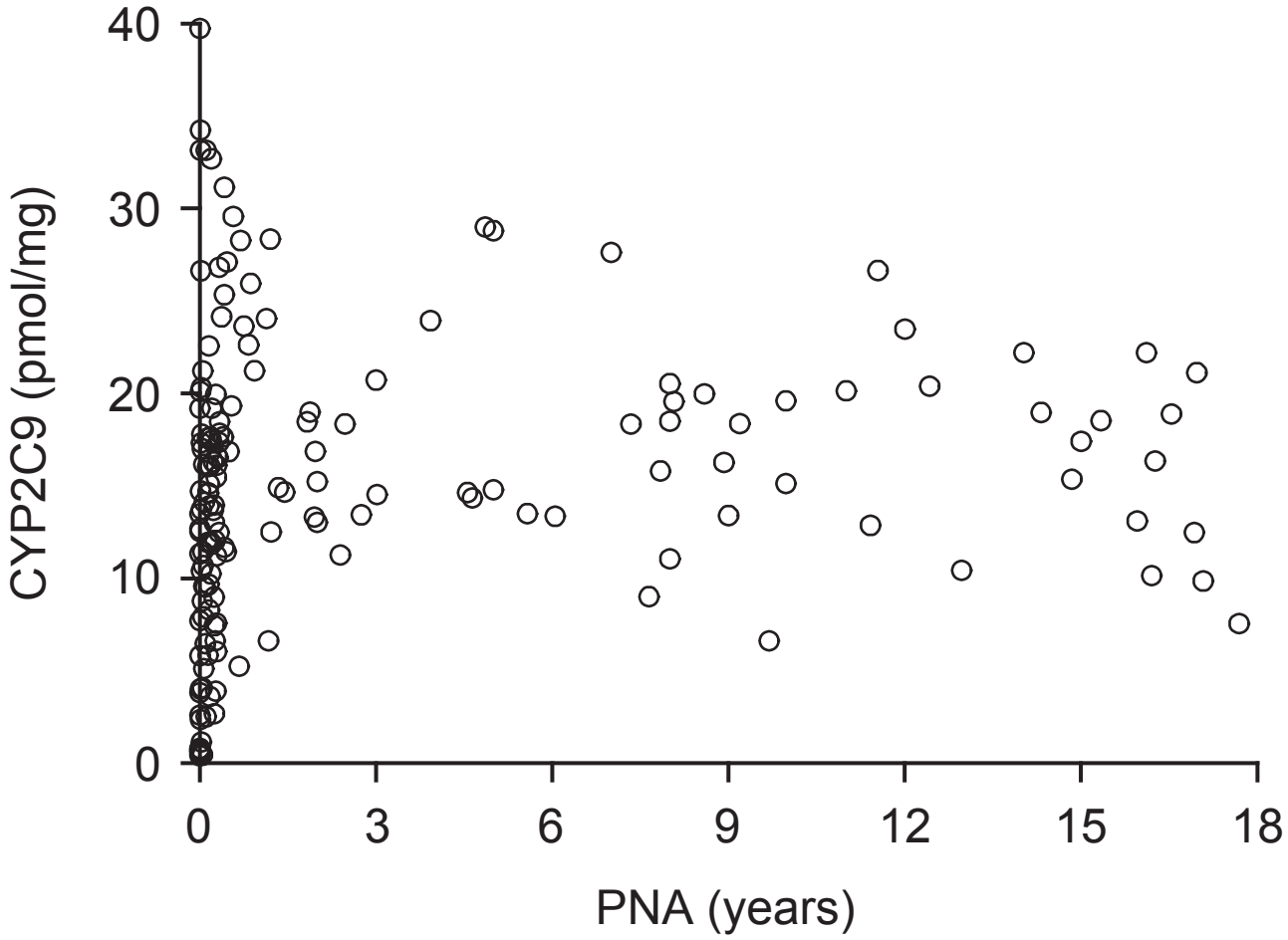

Fig 2B, Koukouritaki et al.

JPET Fast Forward. Published on November 21, 2003 as DOI: 10.1124/jpet.103.060137
This article has not been copyedited and formatted. The final version may differ from this version.

A

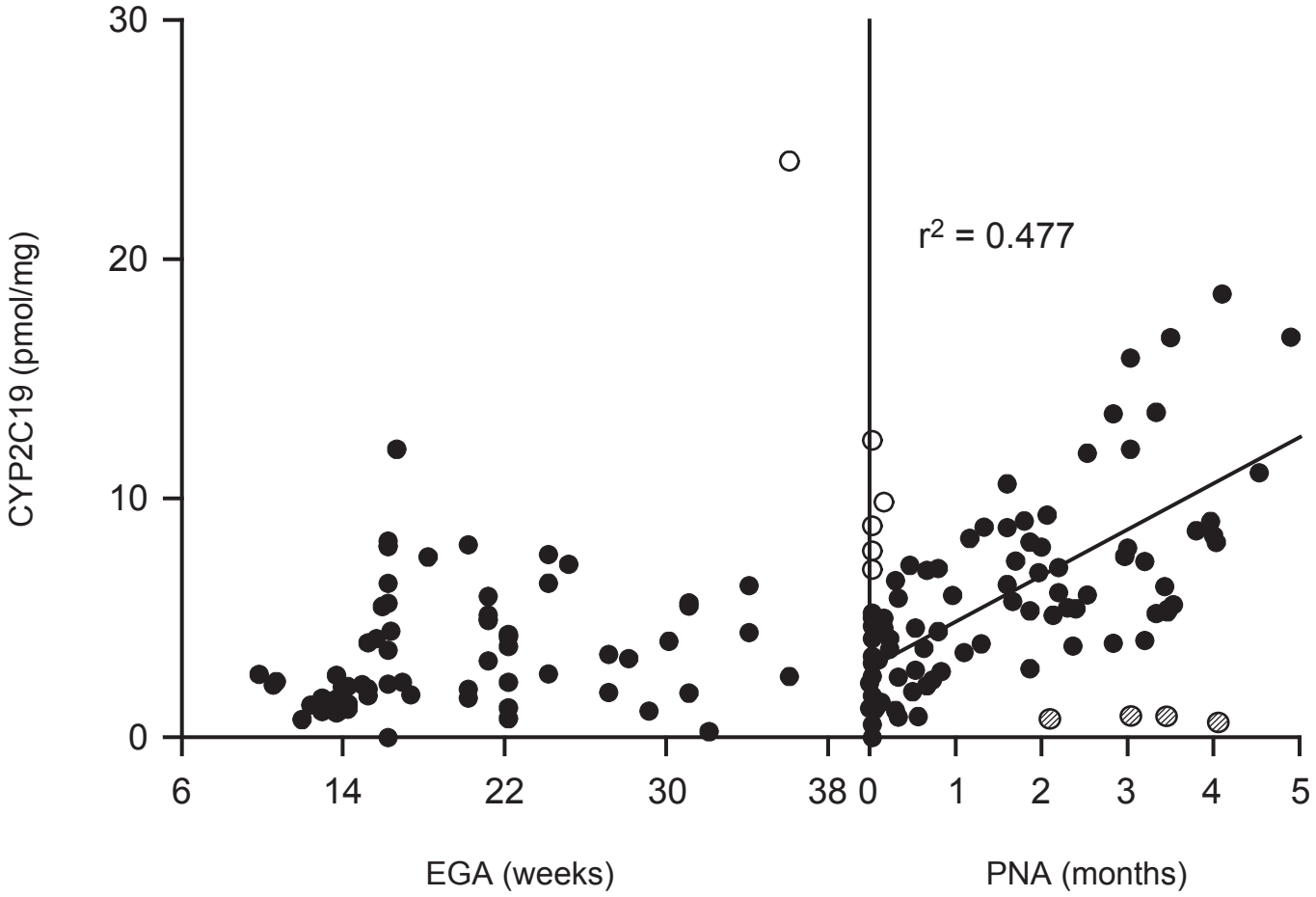

Fig. 3A, Koukouritaki et al.

JPET Fast Forward. Published on November 21, 2003 as DOI: 10.1124/jpet.103.060137
This article has not been copyedited and formatted. The final version may differ from this version.

B

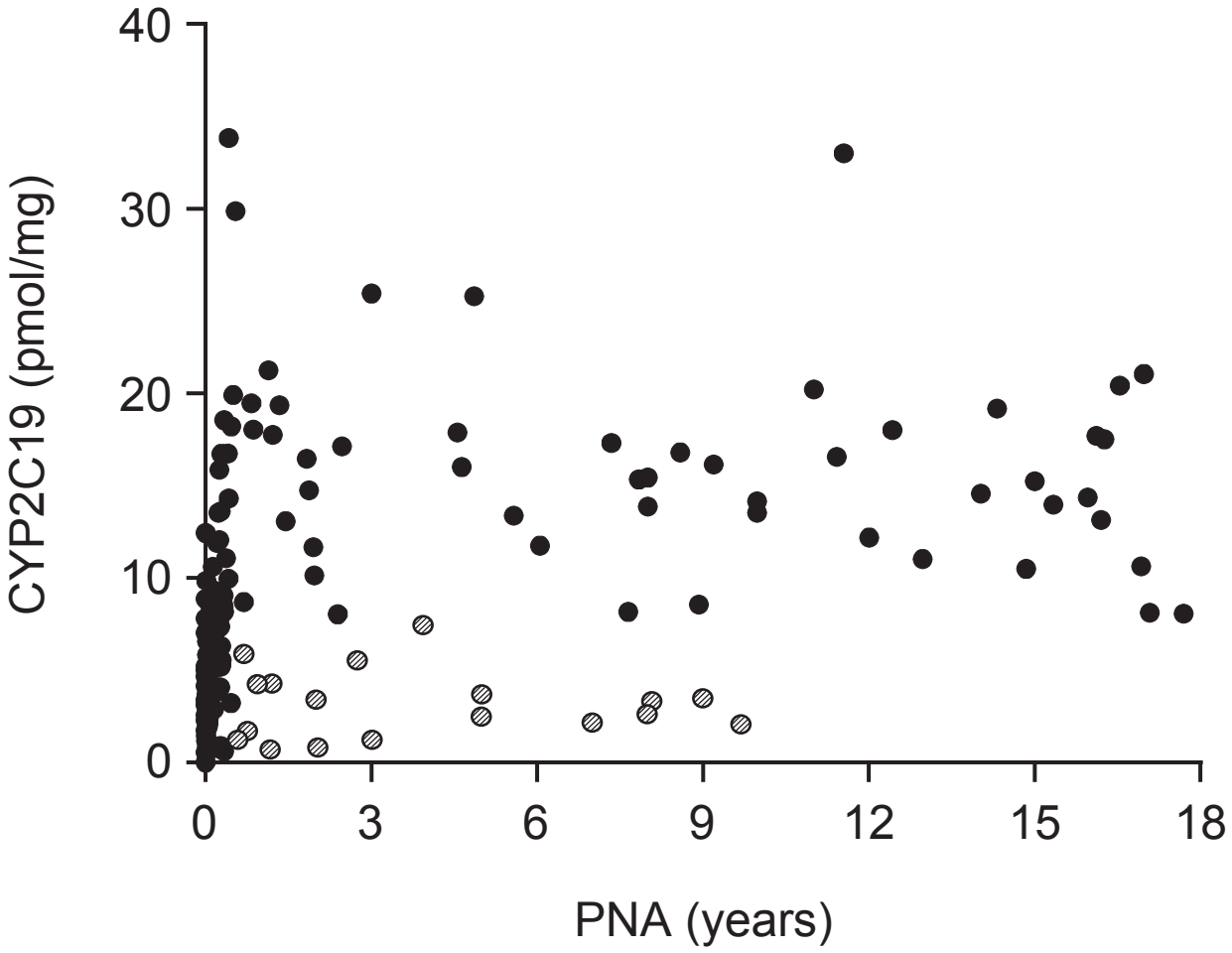

Fig. 3B, Koukouritaki et al.

Figure 5, Koukouritaki et al.

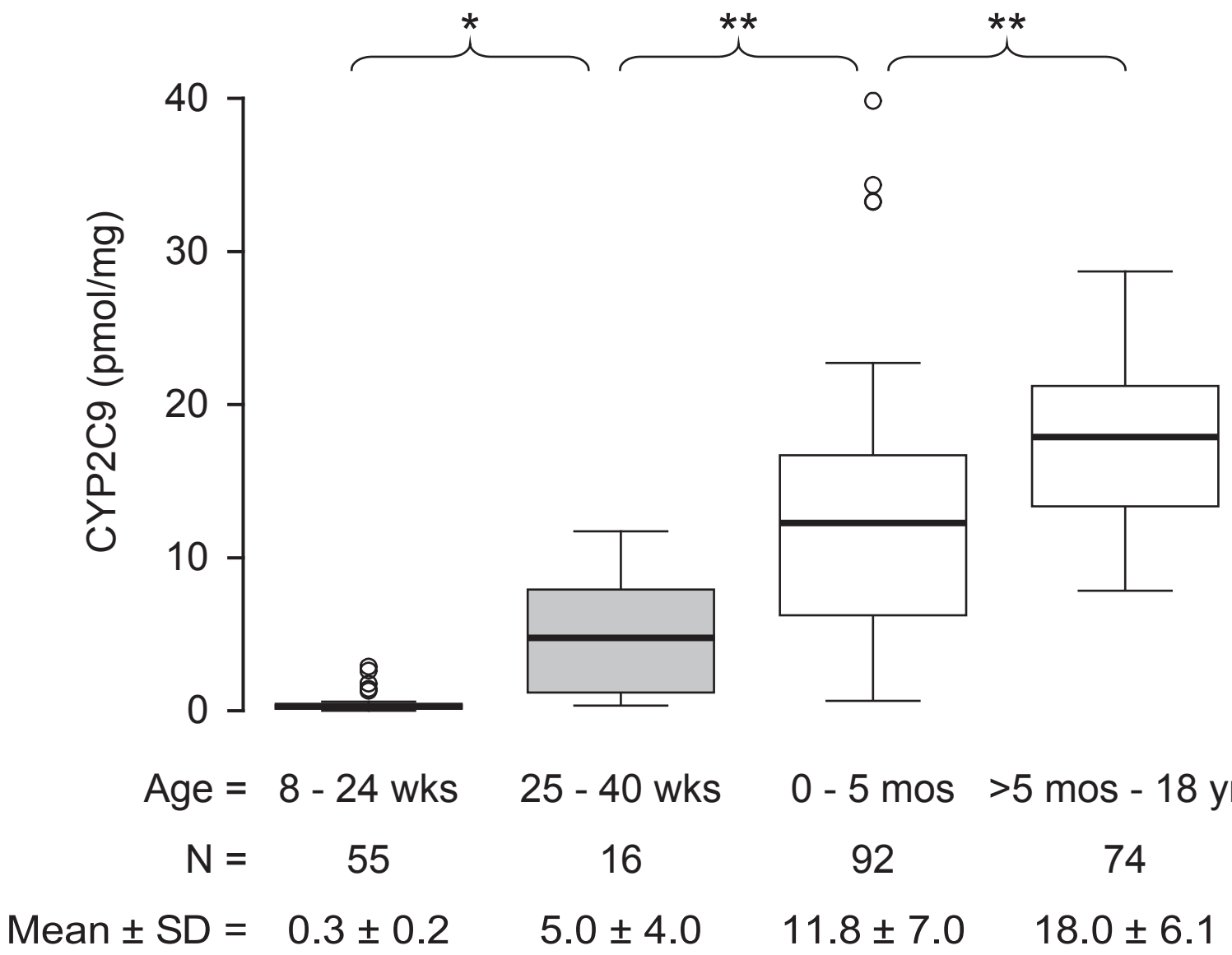

Figure 6, Koukouritaki et al.