

Age-Dependent Human Hepatic Carboxylesterase 1 (CES1) and Carboxylesterase 2 (CES2) Postnatal Ontogeny

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IQR, interquartile range; PPAR, peroxisomal proliferator activated receptor

ABSTRACT

Human hepatic carboxylesterase 1 and 2 (CES1 and CES2) are important for the disposition of ester- and amide- bond containing pharmaceuticals and environmental chemicals. CES1 and CES2 ontogeny has not been well characterized; causing difficulty in addressing concerns regarding juvenile sensitivity to adverse outcomes associated with exposure to certain substrates. To characterize postnatal human hepatic CES1 and CES2 expression, microsomal and cytosolic fractions were prepared using liver samples from subjects without liver disease [N=165, 1d-18 yrs]. Proteins were fractionated, detected and quantitated by western blotting. Median microsomal CES1 was lower among samples from subjects < 3 weeks of age (N=36) compared to the rest of the population (N=126; 6.27 vs 17.5 pmoles/mg microsomal protein, respectively; $p < 0.001$; Kruskal Wallis test). Median cytosolic CES1 expression was lowest among samples from individuals between birth and 3 weeks of age (N=36), markedly greater among those from ages 3 weeks to 6 years (N=90), and then modestly greater still among those over 6 years of age (N=36; median values = 4.7, 15.8, and 16.6 pmoles/mg cytosolic protein, respectively; p values < 0.001 and 0.05, respectively, Kruskal Wallis test). Median microsomal CES2 expression increased across the same three age groups with median values of 1.8, 2.9, and 4.2 pmoles/mg microsomal protein, respectively ($p < 0.001$, both). For cytosolic CES2, only the youngest age group differed from the two older groups ($p < 0.001$; median values=1.29, 1.93, 2.0, respectively). These data suggest that infants < 3 weeks of age would exhibit significantly lower CES1- and CES2-dependent metabolic clearance compared to older individuals.

INTRODUCTION

The human hepatic carboxylesterases (EC 3.1.1.1) are a family of serine esterases that have an important role in the metabolism of numerous chemicals containing ester-, amide-, or thioester-bonds (Sato and Hosokawa 2006). Major pharmaceutical classes include anticoagulants, angiotensin converting enzyme inhibitors, antihyperlipidemic agents, antiviral agents, cancer chemotherapeutics, immunosuppressing compounds, and psychoactive agents, but also several psychoactive drugs of abuse [reviewed in (Laizure *et al.* 2013)]. Environmental chemicals for which the carboxylesterases are important for detoxication and disposition include the organophosphorous (Maxwell 1992) and pyrethroid (Ross *et al.* 2006) insecticides. Of the genes constituting the human carboxylesterase family (Sato and Hosokawa 2006), carboxylesterase 1 (CES1) and carboxylesterase 2 (CES2) are the predominant forms expressed in the liver. These two enzymes share 48% amino acid sequence identity, but exhibit distinct substrate (Sato and Hosokawa 2006) and inhibitor specificities (Parker 2015). Both CES1 and CES2 are found in hepatocyte endoplasmic reticulum and cytosol (Tabata *et al.* 2004; Xu *et al.* 2000). For at least CES1, there is evidence the cytosolic form is missing a putative 18 amino acid N-terminal signal peptide (Tabata *et al.* 2004). Although, the mechanism controlling the dual localization of these enzymes is unknown, there is evidence that processing within the endoplasmic reticulum is necessary to retain activity (Potter *et al.* 1998).

In the report, *Pyrethroids: Evaluation of Data from DNTs and Consideration of Comparative Sensitivity*, the United States Environmental Protection Agency opined that observed age-dependent sensitivity to pyrethroids is largely due to pharmacokinetic factors and in particular, the maturation of required metabolic processes (Document ID: EPA-HQ-OPP-2008-0031-0028 at <http://www.regulations.gov>). However, because of species differences in the major pharmacokinetic factors involved in pyrethroid metabolism, the study of differential sensitivity between juvenile and adult rats will not inform potential human differential sensitivity.

Serum esterases exhibit significant activity toward pyrethroids in the rat, but not the human (Crow *et al.*, 2007). Human, intestinal CES2 is important for the hydrolysis of some, but not all pyrethroids, whereas rat intestinal carboxylesterases exhibit little activity (Crow *et al.* 2007). In the liver, both oxidative and hydrolytic pathways can be important, depending on the pyrethroid, and species differences are observed in the preferred route of metabolism in this organ. Unlike the rat, human CES1 plays a predominant role for several pyrethroids (Ross *et al.* 2006; Godin *et al.*, 2006). Among pyrethroids for which oxidative metabolism is a major contributor, species differences exist in the relative importance of the cytochromes P450 involved. In rats, CYP1A1, CYP1A2, CYP2C6, CYP2C11 and CYP3A1 are the predominant metabolic contributors, whereas in humans, activity is dominated by CYP2C8, CYP2C19 and CYP3A4 (Scollon *et al.* 2009). Thus, for each specific pyrethroid, knowledge of relevant human enzyme developmental trajectory is necessary to inform questions regarding juvenile sensitivity, as well as rationale pediatric drug dosing and avoidance of drug-drug or drug-insecticide interactions.

Considerable information is available regarding the ontogeny of human CYP2C19 (Koukouritaki *et al.* 2004) and CYP3A4 (Stevens *et al.* 2003). In contrast, limited information is available on the ontogeny of CES1 and CES2. Yang *et al.* (2009a) demonstrated a good correlation between human hepatic CES1 and CES2 mRNA and protein, and subsequently showed that the expression of both enzymes was similar to adult levels by 18 yrs of age based on relative mRNA levels. Expression in those equal to or greater than 18 years of age was significantly higher than in individuals between birth and 10 years of age, which in turn was significantly higher than fetal expression. Consistent with this conclusion, Zhu *et al.* (2009) found that human hepatic CES1, but not CES2, protein levels and activity were lower in donors less than 1 year of age versus older samples. However, both of these studies were limited in sample size at critical ages, interpolated protein levels based on mRNA expression using a limited data set to test this correlation, and/or reported relative levels of CES1 and CES2 expression rather than age-dependent CES1 and CES2 specific content. The latter has much

greater utility for extrapolating from *in vitro* determined intrinsic clearance values to *in vivo* age-dependent disposition using modeling, an approach increasingly used to predict disposition among sensitive populations for which ethical constraints prevent direct testing.

The objective of this study was to determine age-dependent changes in postnatal, human hepatic CES1 and CES2 specific content in both the microsomal and cytosolic compartments, to evaluate interindividual variation in expression, and to assess differences in expression as a function of sex and/or ethnicity/race.

MATERIALS AND METHODS

Human Liver Tissue Bank. Anonymized liver tissue samples (N=165), ranging in age from birth to 18 years, were obtained from the Brain and Tissue Bank for Developmental Disorders, University of Maryland, Baltimore. Samples from individuals likely to have experienced liver disease based on the reported cause of death were excluded. A summary of donor demographics are provided in Table 1. Liver microsome and cytosolic fractions were prepared by differential centrifugation as described in Koukouritaki *et al.* (2002) and stored at -80°C until use. The collection and described use of these tissue samples was considered exempt by the Children's Hospital and Health System of Wisconsin Institutional Review Board.

Other Materials. Primary polyclonal antibody raised in rabbit against the carboxy-terminus end of purified human CES1 was obtained from OriGene (Rockville MD) (catalogue no. TA301168). Primary polyclonal antibody raised in rabbit against a conjugated human CES2 peptide sequence corresponding to a region between amino acids 51 and 469 was obtained from Novus Biologicals (Littleton CO) (catalogue no. NBP1-32653). Purified recombinant CES1 protein fused to a polyhistidine carboxy-terminal tag was obtained from Novoprotein (Summit NJ) (catalogue no. C450). Purified recombinant CES2 protein with a carboxy-terminal MYC/DDK

tag was obtained from OriGene (catalogue no. TP303009). Specificity of the primary antibodies was verified by evaluating cross-reactivity of the CES1 and CES2 primary antibodies against the highest concentration of recombinant CES2 and CES1 protein used in the analysis, respectively. Horseradish-conjugated donkey anti-rabbit IgG was obtained from GE Healthcare Lifesciences (Piscataway NJ) (catalogue no. NA934-100UL). Pre-stained protein molecular weight markers were obtained from Invitrogen (Grand Island NY).

Western blot analysis. Aliquots of individual microsomal and cytosolic tissue preparations were fractionated by SDS-polyacrylamide gel electrophoresis along with a range of CES1 and CES2 purified, recombinant protein concentrations and molecular weight standards essentially as described earlier (Koukouritaki *et al.* 2002). Fractionated proteins subsequently were transferred to a nitrocellulose-based membrane (Hybond, GE Healthcare Lifesciences) by electrophoresis. The membrane was incubated with either the CES1 (1:10,000 dilution) or CES2 (1:2,500 dilution) polyclonal antibody, followed by the horseradish peroxidase-conjugated secondary antibody (1:10,000 dilution). Visualization was accomplished using enhanced chemiluminescence (GE Healthcare Lifesciences ECL Plus Detection System) and the ChemiDoc CCD Imaging Analysis system (Bio-Rad, Hercules CA). Linear regression analysis was used to quantify the amount of both CES1 and CES2 protein in each tissue fraction based on the signals observed with the respective purified, recombinant proteins. An $r^2 \geq 0.95$ was accepted as evidence of assay linearity. The interday coefficients of variation for microsomal and cytosolic CES1 measurements were 7.3% and 6.1%, respectively (N=5 replicates, each). The interday coefficients for variation for microsomal and cytosolic CES2 measurements were 3.2 and 4.1% (n= 5 replicates, each).

Statistical Analysis. Scatter plots of protein specific content as a function of age were used to evaluate overall trends. Regression tree analysis using CART version 6 (Salford Systems, San

Diego, CA, USA) was used to evaluate possible age threshold effects. Least average deviation from the median was used to define the tree nodes. Statistical comparisons between age groups were performed using Kruskal-Wallis nonparametric tests, followed by stepwise step-down comparisons of all pairs of data sets (IBM SPSS Statistics 19; SPSS Inc., Chicago, IL). Other variables of interest were compared using nonparametric testing. Linear regression was used to test relationships between continuous variables with the strength of the r^2 (*i.e.*, proximity to 1) and the p value being considered highly relevant. ANOVA and stepwise regression testing were used to assess the multiple factors potentially associated with enzyme content.

RESULTS

Detection of microsomal and cytosolic CES proteins. The developmental expression pattern of the human hepatic CES enzymes was determined by SDS-PAGE and western blot analysis of microsomal and cytosolic samples prepared from a bank of 165 human pediatric liver samples donated by individuals over a wide postnatal age range at the time of death. For CES1, western blotting revealed a single, major immunoreactive band in both the microsomal and cytosolic fractions with an apparent molecular mass near 61 kDa, corresponding to the intact CES1 protein (Figure 1A and 1B) and on re-blotting, an apparent molecular mass near 69 kDa, corresponding to the intact CES2 protein (Figure 1C and 1D). No cross-reactivity between the anti-CES1 antibody and CES2 protein (Figure 1A, Lanes 2-6) or between the anti-CES2 antibody and CES1 protein was observed (Figure 1D, Lanes 13-17).

Overall CES variation and demographic variables. Both CES1 and CES2 were detectable in the majority of samples. With outliers included, microsomal and cytosolic CES1 varied 30- and 70-fold, whereas CES2 varied 11- and 8-fold, respectively. However, without considering extreme values, the overall distribution of CES content for the entire sample varied about 10-

and 6-fold for CES1 and CES2, respectively. There was no relationship between the postmortem interval and the specific content of either CES1 or CES2 (linear regression, each $r^2 \leq 0.02$; each $p > 0.05$). CES1 and CES2 protein levels in both microsomes and cytosol were similar in males and females (data not shown).

When assessed independent of age or any other factors, race/ethnicity was associated with microsomal CES1 and CES2 protein expression (Table 2). Specifically, samples from Caucasians had greater microsomal CES1 expression than those from African Americans, which had greater expression than those from Hispanics ($p \leq 0.05$ and ≤ 0.01 , respectively, Mann Whitney U testing). Similarly, microsomal CES2 expression was significantly greater in both African Americans and Caucasians than Hispanics ($p \leq 0.001$, each comparison; Mann Whitney U testing). Cytosolic CES1 was modestly greater in Caucasians compared to African Americans ($p = 0.05$), whereas no expression differences in cytosolic CES2 were observed.

Human CES1 Ontogeny. CES1 was readily detected in early life hepatic samples, with expression occurring as early as the first day of life in some, but not all, microsomal and cytosolic samples. Complex relationships were observed between age and both microsomal and cytosolic CES1 with a substantial degree of variability across the entire age range (Figures 2A-D).

Based on both classification tree analysis and confirmatory Kruskal Wallis testing, microsomal CES1 activity was lower among samples from subjects younger than 3 weeks of age compared to the rest of the population. This age differential appeared to be driven by the onset of expression during the first 3 weeks of life as no subject older than 18 days of age had non-detectable microsomal CES1 expression (Figures 2A and B). A second node at 6 years was identified by the initial tree analysis, but was not statistically significant ($p = 0.13$) when considered in conjunction with the 3 week node. Thus, samples from individuals between birth and 3 weeks of age [$N = 36$, median value (Interquartile Range; IQR) = 6.27 (4.2-13.4)

pmoles/mg microsomal protein] were lower than those from ages 3 weeks to 6 years [N=91, median value (IQR) = 16.8 (13.2-20.8) pmoles/mg microsomal protein; $p < 0.001$; Kruskal Wallis test, Figure 3], but the CES1 specific content in the later age group was not statistically different from that in the greater than 6 years of age group [N=34, median value (IQR) =18.3 (16.7-21.1) pmoles/mg microsomal protein; $p=0.13$, Kruskal Wallis test]. For cytosolic CES1, expression was much lower among liver samples from individuals between birth and 3 weeks of age [N=36, median value (IQR) = 4.7 (0-8.4) pmoles/mg cytosolic protein, Figure 3] compared to those from ages 3 weeks to 6 years [N=90, median value (IQR)= 15.8 (11.5-20.5) pmoles/mg cytosolic protein; $p < 0.001$; Kruskal Wallis test], which were, in turn, modestly lower compared to those over 6 years of age [N=36, median value (IQR) = 16.6 (14.3-25.3) pmoles/mg cytosolic protein; $p =0.05$; Kruskal Wallis test].

Considering the population as a whole, as CES1 microsomal content increased, there was a corresponding increase in the cytosolic CES1 content (see trend in Figure 3). However, when microsomal and cytosolic CES1 content were compared within individuals, only a modest relationship was observed ($r^2=0.207$, $p<0.001$, linear regression) (data not shown). Both microsomal and cytosolic CES1 was measured in 160/165 samples and of these, the enzyme was detectable in one or the other compartment in 154 samples. Microsomal CES1 content was greater than cytosolic CES1 content in 87/154 samples with a median difference of 5.8 pmol/mg protein (range = 0.1 to 59.5 pmol/mg protein). Cytosolic CES1 content was greater than microsomal CES1 content in 67/154 samples with a median difference of 5.5 pmol/mg protein (range = 0.1 to 63.3 pmol/mg protein). Taken together, these data argue against microsomal content determining cytosolic content and also suggest CES1 content in the two matrices is independently regulated.

Human CES2 Ontogeny. Similar to human CES 1, CES 2 also increased with age with variation apparent across the age range spectrum. The most marked changes occurred during

the first year of life (Figures 4A-4D). Although CES2 was tested using classification tree analysis separately from CES1, the same age nodes were selected for CES2.

Based on both classification tree analysis and confirmatory Kruskal Wallis testing, microsomal CES2 was lower in infants from birth to 3 weeks of age [N=36; median value (IQR) = 1.8 (1.6-2.5) pmoles/mg microsomal protein] compared to those between 3 weeks and 6 yrs of age [N=91; median value (IQR) = 2.9 (2.1-3.7) pmoles/mg microsomal protein; $p < 0.001$, Kruskal Wallis test, Figure 5]. The middle age group, those older than 3 weeks but less than or equal to 6 yrs of age, had values which were, in turn, lower than values in the oldest group, children over age 6 [N=34; median value (IQR)=4.2 (2.7-5.2) pmoles/mg microsomal protein $p < 0.001$, Kruskal Wallis test]. Cytosolic CES2 content was lower in samples from infants from birth to 3 weeks of age [N=36; median (IQR) = 1.29 (0-1.53) pmoles/mg microsomal protein, Figure 5] compared to the two older age groups [N=91 and N=34, 1.93 (1.3-2.1) and 2.0 (1.4-2.5), respectively ($p < 0.001$, each comparison; Kruskal Wallis test)]. However, the two older age groups did not differ in cytosolic CES2 content ($p=0.26$).

Similar to human CES1, when comparing the amount of microsomal and cytosolic CES2 in individual samples, there was a statistically significant, but numerically modest relationship ($r^2=0.08$, $p < 0.001$, linear regression) (data not shown). Microsomal and cytosolic CES2 was measured in 157/165 samples and of these, was detectable in one or the other compartment in 154 samples. Microsomal CES2 content was greater than cytosolic content in 133/154 samples with a median difference of 1.42 pmol/mg protein (range = 0.03 to 6.24 pmol/mg protein). Cytosolic CES2 content was greater than microsomal content in only 21/154 samples with a median difference of 0.84 pmol/mg protein (range = 0.03 to 11.60 pmol/mg protein).

Simultaneous Consideration of Factors Impacting CES Expression. Considering age category, sex and race/ethnicity simultaneously, only the variable "age category" remained significantly associated with human CES1 content in both the microsomal and cytosolic

compartments ($p < 0.001$, each model; ANOVA). Cytosolic CES2 was similar, *i.e.*, only the age categorization was significantly associated ($p < 0.001$, ANOVA). For microsomal CES2, the relationship between enzyme content and sex was ambiguous ($p = 0.08$, ANOVA) when age was considered simultaneously. Nevertheless, the influence of age on CES2 enzyme content continued to be highly significant with sex considered simultaneously ($p < 0.001$, ANOVA). Of note, with age group considered simultaneously, race/ethnic group was no longer significantly associated with CES1 or CES2, in either compartment.

DISCUSSION

The results of this project confirm that human hepatic microsomal and cytosolic CES1 and CES2 expression is developmentally regulated based on the strong association between postnatal age and quantitatively measured protein values. For both enzymes, expression was markedly lower among infants 3 weeks of age or less compared to older infants and children. Of note, for microsomal CES1, all samples exhibited some expression after three weeks, whereas the universal onset of CES2 expression appeared somewhat later or its expression was suppressed by an unidentified mechanism in a small number of samples. After three weeks of age, ongoing developmental changes varied by enzyme and by compartment. Microsomal CES1 and cytosolic CES2 did not exhibit additional age related differences. In contrast, for microsomal CES2 and cytosolic CES1, the samples from children over the age of six exhibited statistically significantly greater expression than those between 3 weeks and 6 years of age. This suggests that significant developmental changes continue during the window between 3 weeks and 6 years. This may be particularly relevant for CES1 given its high expression levels in the liver. However, the rate of change during this time appears modest as reflected in the relatively small numerical change over a wide time interval in comparison to the relatively steep surge in expression that occurs during the first three weeks after birth.

Race/ethnicity appeared to be associated with differences in enzyme expression in univariate testing (Table 2), but when this was evaluated with multivariate testing that included age, it was no longer significant; age was the only factor that entered into the model.

The above conclusions are consistent with literature reports that have evaluated changes in CES1 and CES2 mRNA levels during early life stages (Yang *et al.* 2009a), protein expression and activity data (Yang *et al.* 2009a; Zhu *et al.* 2009). However, the limited data sets used in these earlier studies prevented a precise determination of the developmental trajectory and neither study differentiated between carboxylesterase content in the microsomal and cytosolic compartments. Yang *et al.* (2009a) demonstrated significant differences in mRNA content and activity only between fetal (12 to 32 weeks), children (birth to 10 years) and adults (≥ 18 years), while Zhu *et al.* (2009) were able to demonstrate combined hepatic microsomal and cytosolic CES1, but not CES2, was significantly lower in tissue samples from individuals less than 1 year of age compared to older age groups. This contrasts to the data presented herein which demonstrates a significant difference between infants less than 3 weeks of age and older children. These data, combined with those from Yang *et al.* (2009a) and Zhu *et al.* (2009) convincingly document that both human hepatic microsomal and cytosolic CES1 and CES2 belong to the class 3 group of enzymes involved in drug and toxicant disposition, *i.e.*, they exhibit low to no expression in the fetus and that expression increases substantially during the first months to 2 years of life (Hines 2012).

Over the entire population, microsomal and cytosolic CES1 varied about 30- and 70-fold, respectively, whereas microsomal and cytosolic CES2 varied about 11- and 8-fold, respectively. In both compartments, the median CES1 value varied by about 3- to 3.5-fold across the three age groups, whereas the median CES2 value varied by 1.5- to 2.5-fold. Importantly, the within age bracket variation was substantive. We speculate that this within age bracket variability is likely explained in part by genetic variation. Six *CES1* single nucleotide polymorphisms have been identified that, *in vitro*, exhibit various degrees of decreased activity or increased promoter

activity, or in vivo, are associated with altered drug disposition. Three *CES2* single nucleotide polymorphisms have been associated with decreased activity both in vitro and in vivo (reviewed in Merali *et al.* 2014). However, whether any of these genetic variants function through altered protein levels versus changes in enzyme activity is unknown. The *CES1* rs3785161 variant that is associated with increased promoter activity (Geshi *et al.* 2005) and the *CES1* rs2241409 variant that is associated with decreased transcript levels (Marsh *et al.* 2004) would be expected to alter enzyme expression levels, but this has not been shown directly. Exposures to environmental factors may also have contributed to the observed variability. Inflammatory cytokines, such as IL6, have been shown to repress *CES1* and *CES2* expression through a distal regulatory element (Yang *et al.* 2007) and a nuclear factor-erythroid 2 related factor element at *CES1* position –2025 mediates increased expression in response to oxidative stress (Maruichi *et al.* 2010). Finally, Ghosh and Natarajan (2001) identified three functional peroxisomal proliferator response elements at *CES1* positions –176, –779 and –1316 that mediate transcriptional repression by ligand-activated peroxisomal proliferator activated receptor (PPAR) α or PPAR γ . Although the pregnane X receptor is involved in the induction of many genes encoding xenobiotic metabolizing enzymes, this receptor is not involved in regulating *CES1* or *CES2* expression (Yang *et al.* 2009b).

This study is limited by the absence of *CES1* and *CES2* activity data for each sample. Such data is desirable if highly specific substrates are available and assays can be developed with sufficient sensitivity to allow an adequate signal to noise ratio with the small amount of protein available for each sample in the tissue bank; this was not the case for these enzymes. However, past experiments performed to evaluate the developmental trajectories of other enzymes employing this same tissue bank and where highly sensitive and specific activity assays were available demonstrated excellent correlation between enzyme specific content and specific activity (Koukouritaki *et al.*, 2004; Li *et al.*, 2012). This past experience provides some confidence that a similar relationship would be observed for *CES1* and *CES2*. A second

limitation is that the sample set did not include tissue from adults (>18 years of age) as a comparator. Indeed, because the tissue bank was designed to have maximum power to detect differences at younger ages, the median age of the entire sample set was 3.5 months and of the samples >3 weeks of age, 9.6 months. Thus, one might expect the reported median CES1 and CES2 protein levels in the greater than 3 weeks age bracket to be less than one would observed in adults greater than 18 years of age. Consistent with this expectation, Shi *et al.* (2011) reported an approximate 2-fold increase in mean relative CES1 protein levels in hepatic S9 fractions from donor samples between 5 weeks and 6.5 months of age and adults (>18 years of age). Similar results have recently been reported for CES2 (Chen *et al.* 2015).

The data from this project will be used in conjunction with available data on the ontogeny of other relevant enzymes and numerous physiologic variables to develop high quality physiologically-based pharmacokinetic models for specific CES1 and CES2 substrates. Importantly, because of the somewhat unique dual compartment localization of the hepatic CES enzymes, such models should consider the contribution of both compartments to clearance. The anticipated simulations will be valuable for therapeutics, but will be critical for assessing the safety of compounds such as the pyrethroids and pyrethrins which cannot be directly studied in children. As such, these new data represent a significant advance over existing data which were insufficient for this purpose both because of the poor time resolution and use of relative units of measure.

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AUTHORSHIP CONTRIBUTION:

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FOOTNOTES:

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FIGURE LEGENDS

Fig.1. Western blot analysis of human CES1 and CES2 in pediatric hepatic microsomal samples. (A) Western blot with anti-CES1 antibody; Lane 1, molecular weight standards as shown (kDa); Lanes 2-6, purified recombinant CES2 standards (6.5, 12, 25, 50 and 100 ng); Lanes 7-18, microsomal hepatic protein samples (10 µg each). (B) Western blot with anti-CES1 antibody; Lanes 1-12, microsomal hepatic protein samples (10 µg each); Lanes 13-17, purified recombinant CES1 standards (12.5, 25, 50, 100, 150 ng); Lane 18, molecular weight standards as shown (kDa). (C) Western blot with anti-CES2 antibody; Lane 1, molecular weight standards as shown (kDa); Lanes 2-6, purified recombinant CES2 standards (6.5, 12, 25, 50 and 100 ng); Lanes 7-18, microsomal hepatic protein samples (10 µg each). (D) Western blot with anti-CES2 antibody; Lanes 1-12, microsomal hepatic protein samples (10 µg each); Lanes 13-17, purified recombinant CES1 standards; Lane 18, molecular weight standards as shown. Microsomal samples from differing age groups were loaded in a non-ordered fashion.

Fig. 2. The relationship between human CES1 and age in postmortem microsomal and cytosolic liver samples. (A) Overall relationship with microsomal CES1 in samples from birth to 18 years (N=161). The two added vertical lines represent the two nodes, age 3 weeks (left line) and 6 years (right line), selected by classification regression tree analysis as indicative of appropriate age groupings. The three week age classification (solid line) was confirmed on Kruskal Wallis statistical testing, whereas the 6 year grouping (indicated by a dotted line) was not. (B) Relationship between microsomal CES1 and age in liver samples from the subset of subjects less than one year of age (N = 102). The added solid vertical line represents the 3 week time point selected by classification trees and confirmed by statistical testing as appropriate age stratification. (C) Relationship between cytosolic CES1 and age in postmortem cytosolic liver samples from birth to 18 years (N=162). The vertical lines represent the two nodes selected by

classification trees as indicative of appropriate age groupings: 3 weeks (left line) and 6 years (right line). Both were confirmed as statistically significant using Kruskal Wallis testing. (D) Relationship between cytosolic CES1 and age in postmortem cytosolic liver samples from a subset of subjects less than one year of age (N=101). The vertical line represents the time point selected by classification trees as an appropriate age grouping (3 weeks).

Fig. 3. Summary of microsomal (white boxes) and cytosolic (gray boxes) human CES1 developmental expression pattern. CES1 specific content as a function of age was grouped using classification tree analysis to minimize differences within while maximizing differences between age brackets. The resulting data are shown as box and whisker plots in which the horizontal bar represents median CES1 content, boxes the upper and lower quartiles, and vertical bars the 5th to 95th percentiles. Outliers, defined as having specific contents outside 1.5 times the 25th to 75th percentiles are shown as open circles, but were excluded from the analyses except for determining absolute ranges in expression. The youngest age group differed significantly from the other two for both matrices ($p < 0.001$, each comparison; Kruskal Wallis testing), whereas the middle age group was modestly significantly different from the older group in CES1 cytosolic content ($p=0.05$), but did not differ in CES1 microsomal content ($p=0.13$).

Fig. 4. The relationship between human CES2 and age in postmortem microsomal and cytosolic liver samples. (A) The relationship between microsomal CES2 and age in postmortem liver samples from donors ages birth to 18 years (N=161). The vertical lines represent the two nodes selected by classification trees as indicative of appropriate age groupings: 3 weeks (left line) and 312 weeks, or 6 years (right line). Both were confirmed as statistically significant using Kruskal Wallis testing. (B) The relationship between microsomal CES2 and age in postmortem liver samples from the subset of subjects less than one year of age (N=102). The vertical line

represents the time point selected by classification trees as an appropriate age grouping (3 weeks). (C) Relationship between cytosolic CES2 and age from postmortem liver samples (N = 162). The two vertical lines at 3 weeks (left) and 6 years (right) of age represent the nodes selected by classification trees. The three week age classification (solid line) was confirmed on Kruskal Wallis statistical testing (see below), whereas the 6 year grouping (indicated by a dotted line) was not. (D) Relationship between cytosolic CES2 and age in postmortem liver samples from a subset of subjects less than one year of age (N= 101). The vertical line at three weeks represents the time point selected by classification trees and statistically confirmed (Kruskal Wallis) as an appropriate age grouping.

Fig. 5. Summary of microsomal (white boxes) and cytosolic (gray boxes) human CES2 developmental expression pattern. CES2 specific content as a function of age was grouped using classification regression trees to minimize differences within age groups while maximizing differences between age brackets. The resulting data are shown as box and whisker plots in which the horizontal bar represents median CES2 content, boxes are the upper and lower quartiles, and vertical bars the 5th to 95th percentiles. Outliers, defined as having specific contents outside 1.5 times the 25th to 75th percentiles are shown as open circles, but were excluded from the analyses except for reporting absolute expression ranges. The youngest age group differed significantly from the other two for both matrices ($***p < 0.001$, each comparison, Kruskal Wallis testing) whereas the middle age group was significantly different from the older group in CES1 microsomal content ($p < 0.001$), but did not differ in CES1 cytosolic content ($p = 0.26$).

Table 1: Tissue Sample Donor Demographics

Variable		Median	Range
Age at death (mos)		3.7	0-212
Postmortem Interval (hrs)		17	1-41
		N	% of Total
Hepatic Tissue Samples		165 ¹	100
Sex	Male	104	63
	Female	58	35
	Unknown	3	2
Ethnicity/Race	Northern European Caucasian	79	48
	African American	62	38
	Hispanic	16	10
	Asian	2	1
	Native American	1	<1
	Unknown	5	3

¹ To ensure good age representation and adequate power, target sample sizes for birth to 30 days, greater than 30 days to 1 year, greater than 1 year to 5 years, greater than 5 years to 10 years, and greater than 10 years to 18 years age brackets were developed based on data from the existing literature on drug and toxicant metabolizing enzyme ontogeny. Samples sizes were sufficient to provide at least 80% power to detect a 1 standard deviation change in enzyme specific content between age brackets assuming $\alpha=0.05$.

Table 2. Immuno-detected CES content from postmortem fractionated hepatic samples^{1,2}

Enzyme	African Americans N=62	Caucasians N=79	Latinos N=16	Overall <i>p</i>
Microsomal CES 1	14.99* (9.62-18.51)	17.95 (13.60-20.67)	10.86** (4.33-20.72)	0.01
Cytosolic CES1	13.62* (5.51-16.91)	15.46 (0.35-19.58)	11.94 (0-23.21)	0.155
Microsomal CES2	2.79 (2.05-3.98)	2.99 (2.20-3.93)	1.79*** (1.52-1.98)	0.001
Cytosolic CES2	1.83 (1.22-2.23)	1.69 (1.13-2.07)	1.51 (0-2.12)	0.23

¹ Data are given in median pmoles/mg protein (interquartile range).

² Each enzyme is compared across the three donor race/ethnicity groups. The overall *p* value was derived from Kruskal Wallis comparison across the three groups, whereas the asterisk *p* value designations within the table represent two way comparisons using the Mann Whitney U test.; * $p \leq 0.05$, compared to Caucasians; ** $p \leq 0.01$, compared to the other two groups; *** $p \leq 0.001$, compared to the other two groups

Figure 1

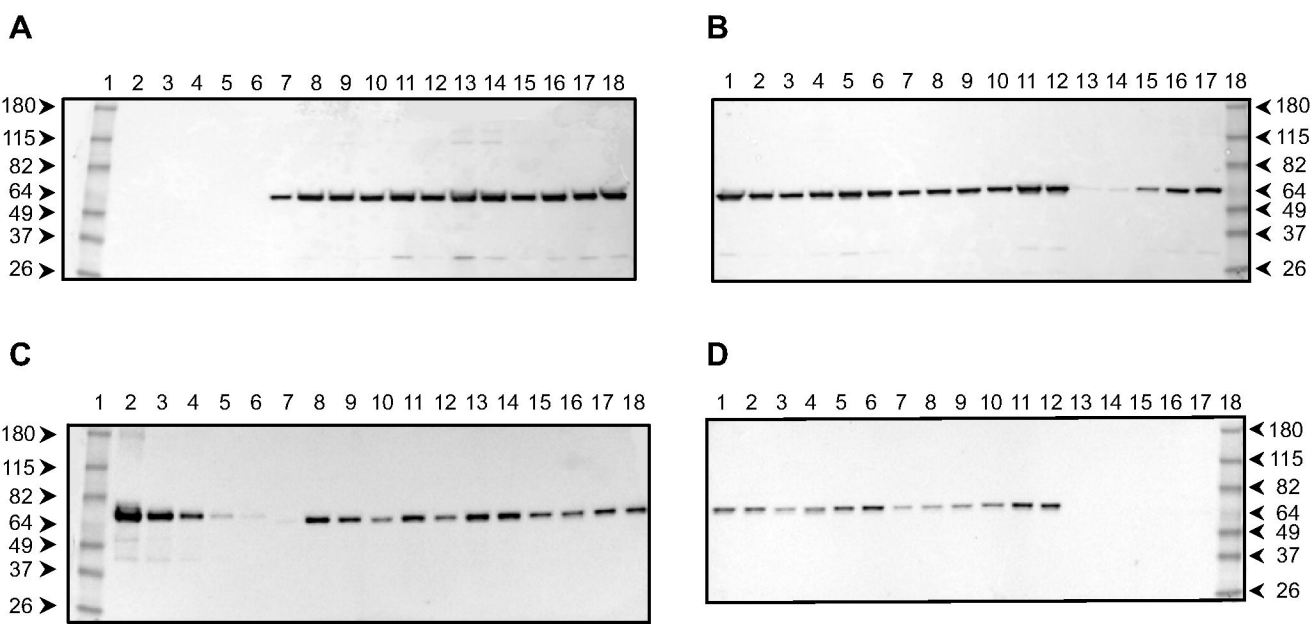


Figure 2

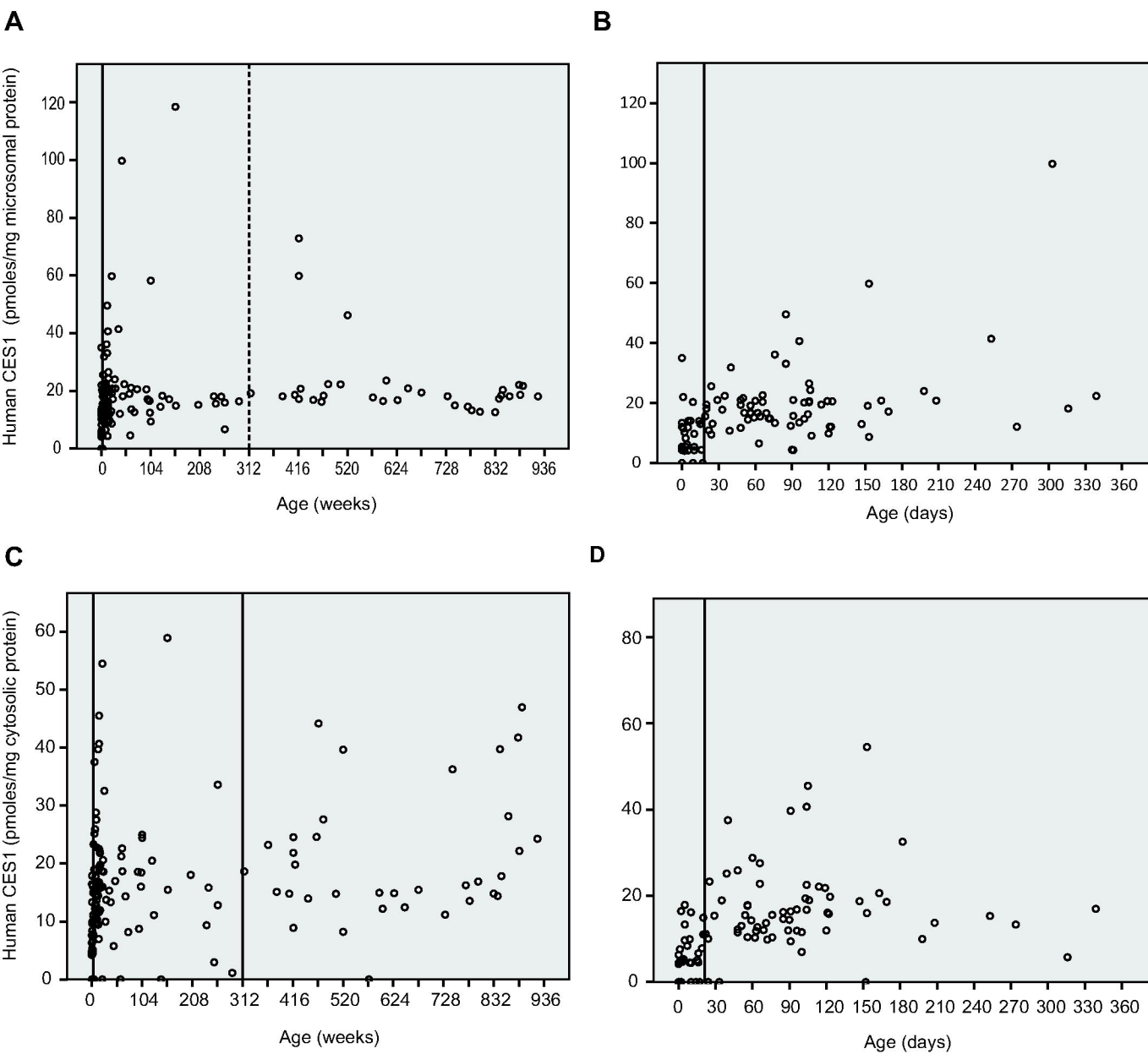


Figure 3

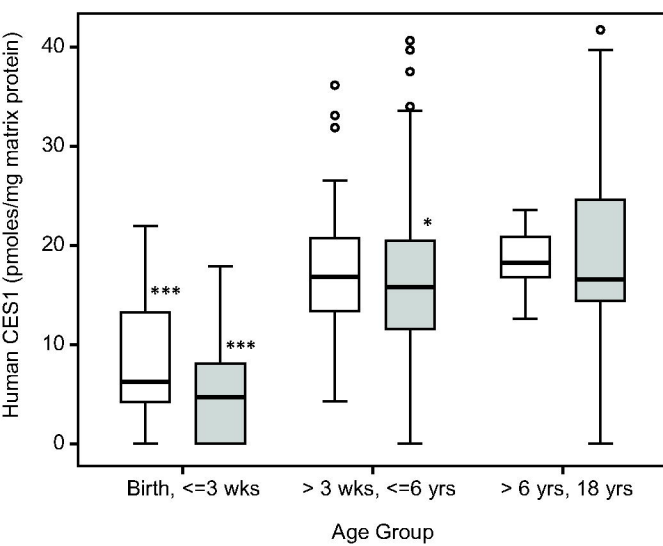


Figure 4

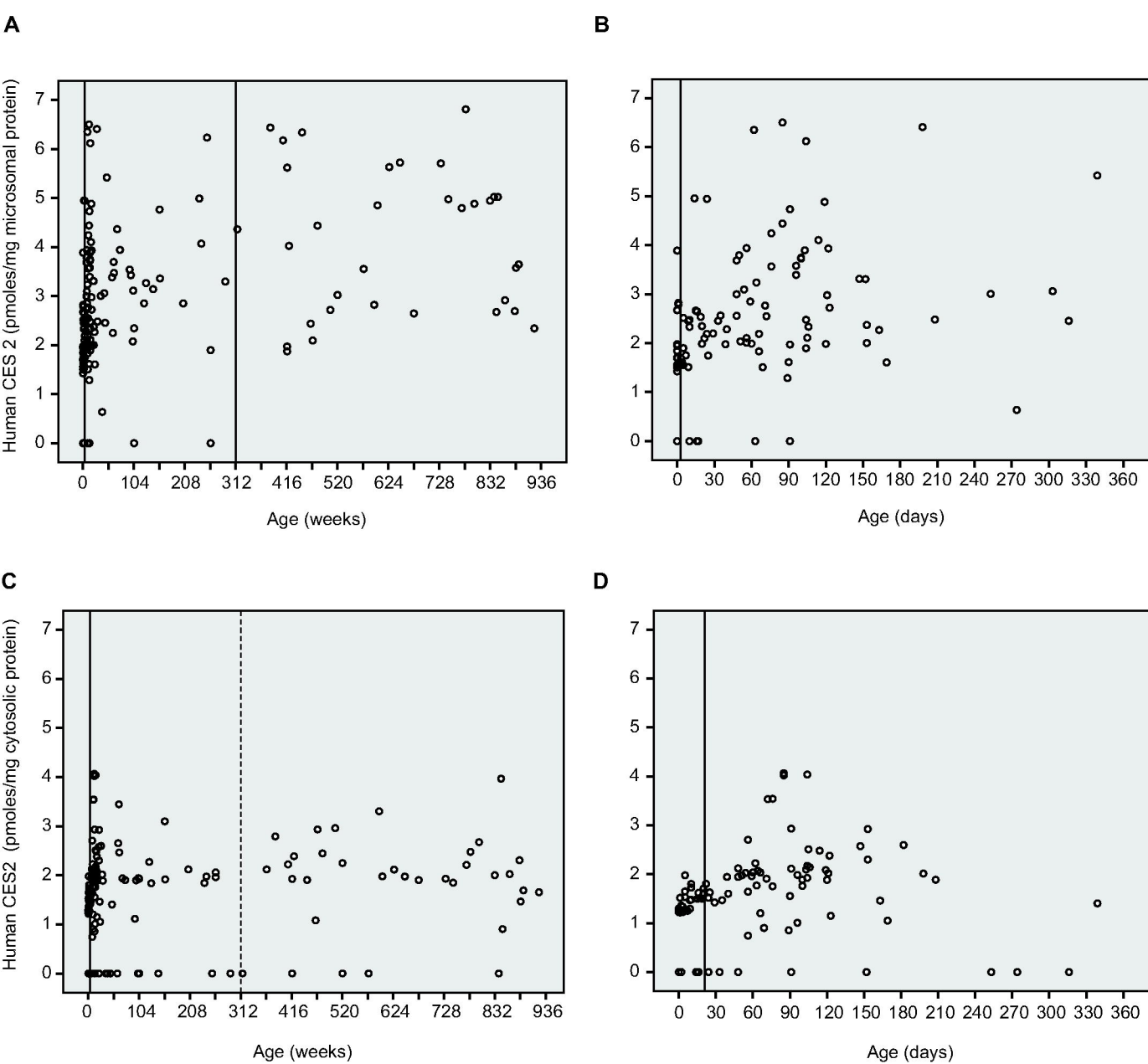


Figure 5

