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Title Page

Developmental Expression of CYP2B6: A Comprehensive Analysis of mRNA Expression, Protein Content and Bupropion Hydroxylase Activity and the Impact of Genetic Variation

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Abbreviations: HPLC, high performance liquid chromatography

Abstract

Although CYP2B6 catalyzes the biotransformation of many drugs used clinically in children and adults, information regarding the effects of development on CYP2B6 expression and activity are scarce. Utilizing a large panel of human liver samples (201 donors: 24 fetal, 141 pediatric and 36 adult), we quantified CYP2B6 mRNA and protein expression levels, characterized CYP2B6 (bupropion hydroxylase) activity in human liver microsomes (HLMs) and performed an extensive genotype analysis to differentiate *CYP2B6* haplotypes so that the impact of genetic variation on these parameters could be assessed. Fetal livers contained extremely low levels of CYP2B6 mRNA relative to post-natal samples and fetal HLMs did not appear to catalyze bupropion hydroxylation, but fetal CYP2B6 protein levels were not significantly different from post-natal levels. Considerable inter-individual variation in CYP2B6 mRNA expression, protein levels and activity was observed in post-natal HLMs (mRNA, ~40,000-fold; protein, ~300-fold; activity, ~600-fold). The extremely wide range of inter-individual variability in CYP2B6 expression and activity was significantly associated with age (p<0.01) following log transformation of the data. Our data suggest that CYP2B6 activity appears as early as the first day of life, increases through infancy, and by 1 year of age, CYP2B6 levels and activity may approach those of adults. Surprisingly, CYP2B6 inter-individual variability was not significantly associated with genetic variation in *CYP2B6,* nor with differences in gender or ethnicity, suggesting that factors other than these are largely responsible for the wide range of variability in CYP2B6 expression and activity observed among a large group of individuals/samples.

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Introduction

CYP2B6 is one of about 15 cytochrome P450 enzymes that are primarily responsible for the oxidation of drugs and other xenobiotics and is the only known functional member of the CYP2B subfamily in humans (Wang and Tompkins, 2008; Zanger and Klein, 2013). CYP2B6 was once believed to play an insignificant role in drug metabolism because it was initially reported to be present in low levels and expressed in only a small proportion of livers (Mimura et al., 1993). More recent studies have demonstrated that CYP2B6 is present in most post-natal livers (Croom et al., 2009; Lamba et al., 2003; Stresser and Kupfer, 1999) and has a relative abundance varying from 1 to 10 percent of the total CYP content (Wang and Tompkins, 2008). Today, CYP2B6 is known to catalyze the biotransformation of over 90 substrates (Wang and Tompkins, 2008) including clinically used drugs such as the antineoplastic agent, cyclophosphamide (Roy et al., 1999), the anti-smoking cessation drug, bupropion (Faucette et al., 2000), the anesthetic, propofol (Court et al., 2001), and the antiretroviral, efavirenz (Desta et al., 2007; Ward et al., 2003) as well as other compounds including drugs of abuse, pesticides and environmental toxins. A few reactions, such as bupropion hydroxylation and efavirenz 8-hydroxylation, are almost exclusively catalyzed by CYP2B6 and have been used to assess CYP2B6 activity both *in vitro* and *in vivo*.

Previous studies noted marked inter-individual variability in CYP2B6 expression and activity in human livers; *in vitro* CYP2B6 mRNA expression (Hofmann et al., 2008; Lamba et al., 2003), protein levels (Hesse et al., 2004) and activity (Croom et al., 2010) have all been reported to exceed 250-fold. This remarkable variation in CYP2B6 expression and activity has the potential to result in therapeutic and toxic responses to medications metabolized by this enzyme, particularly for those drugs with narrow therapeutic indices. Hence, it is imperative that the sources responsible for the observed variability in CYP2B6 expression and activity be identified. Numerous investigations have attempted to elucidate the mechanisms responsible for the variability in CYP2B6 expression and activity, examining factors such as age, gender, ethnicity, genetic polymorphisms and chemical induction. In adults, CYP2B6 expression and activity appear to be determined largely by genotype and chemical regulation.

CYP2B6 is one of the most polymorphic CYP genes in humans with numerous single nucleotide polymorphisms encoding 38 CYP2B6 protein variants to date (http://www.cypalleles.ki.se/; accessed

September 4, 2015). The functional impact of these allelic variants is complex. At least 12 alleles have been associated with no or low activity, but these alleles appear to be rare in the populations investigated (Zanger and Klein, 2013). SNPs associated with *CYP2B6* allelic variants can result in protein expression levels that may be increased, decreased or exhibit no change. The functional consequences of allelic variation on CYP2B6 activity *in vitro* have been shown to be allele, substrate and expression-system dependent (Zanger and Klein, 2013). CYP2B6 is strongly inducible by several drugs including the "classical" inducers rifampicin and phenobarbital, which regulate CYP2B6 expression through the constitutive androstane and pregnane X receptors CAR and PXR (Faucette et al., 2007; Lamba et al., 2004). Induction of CYP2B6 activity in cultured hepatocytes treated with phenobarbital has been shown to be as high as 32-fold (Yajima et al., 2014).

Although developmental age has been shown to have an impact on the expression of other CYPs (Hines, 2007; 2008; 2013; Hines and McCarver, 2002), little information exists regarding the effects of development on CYP2B6 expression and activity. Only a few studies have examined the effects of age on CYP2B6 expression and almost all of these studies are hampered by the inclusion of only a small number of fetal or pediatric samples. The most comprehensive study of CYP2B6 developmental changes to date characterized CYP2B6 protein levels in 217 fetal and pediatric liver samples from donors ranging from 10 weeks gestation to 17 years of age (Croom et al., 2009). A two-fold increase in median CYP2B6 protein expression was observed in those samples outside the neonatal period (birth to 30 days postnatal) compared with fetal and neonatal samples, but CYP2B6 expression did not vary significantly with gender, *CYP2B6*6* genotype or post-mortem interval.

The current study was undertaken to explore in greater detail factors involved in CYP2B6 expression and activity, with particular regard to the contributions of age and genetic variation. Utilizing a collection of liver samples from 201 donors that spanned a wide range of ages (~11 weeks gestation to 79 years of age), the current study quantified CYP2B6 protein expression in human liver microsomes like previous studies, but also characterized CYP2B6 mRNA expression levels, CYP2B6 bupropion hydroxylase activities in human liver microsomes and performed an extensive genotype analysis to differentiate *CYP2B6* haplotypes so that the impact of genetic variation on these parameters could be assessed. In

addition, polymorphisms in genes that may impact CYP2B6 expression and activity were also investigated.

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Materials and Methods

Materials and Reagents

Bupropion and hydroxybupropion were purchased from Toronto Research Chemicals (Toronto, ON, Canada). EDTA, formic acid, glucose-6-phospate, glucose-6-phosphate dehydrogenase, and ß-NADP were purchased from Sigma Aldrich Chemical Co. (St. Louis, MO, USA). Acetonitrile (OPTIMA®grade) and triethylamine were purchased from Thermo-Fisher Scientific (Fairlawn, NJ, USA). Criterion XT Bis-Tris 10% gels, XT Sample Buffer, XT Reducing Agent, XT MOPS running Buffer, 10X Tris/Glycine transfer buffer, Kaleidoscope Protein Standard, and thin filter paper were purchased from Bio-Rad Life Science (Hercules, CA, USA). Donkey anti-rabbit IgG HRP conjugate, ECL PlusWestern blotting detection reagents and analysis system, Hyperfilm ECL and Hybond ECL nitrocellulose membrane were purchased from GE Healthcare (Piscataway, NJ, USA). Microsomes from baculovirus-infected insect cells expressing human CYP2B6 (SUPERSOMES^{TM}) and a polyclonal rabbit anti-human CYP2B6 antibody were purchased from BD Gentest (Woburn, MA, USA). Pooled human liver microsomes (n=16 donors, mixed gender, Lot No. 0210127) were a generous gift from Xenotech LLC (Lenexa, KS, USA). All genotyping and gene expression assays were purchased from Applied Biosystems (Foster City, CA, USA).

Liver Samples

A total of 201 prenatal and postnatal liver samples were included in this study. The use of these tissues was classified as nonhuman subjects research by the University of Missouri-Kansas City Pediatric Health Sciences Review Board (MO, USA). All prenatal and some postnatal tissue samples were obtained through the National Institute of Child Health and Human Development-supported tissue retrieval programs at the Central Laboratory for Human Embryology at the University of Washington (Seattle, WA, USA), the Brain and Tissue Bank for Developmental Disorders at the University of Maryland (Baltimore, MD, USA) and the NIH-supported Liver Tissue Cell Distribution System (LTCDS) at the University of Minnesota and the University of Pittsburgh. Additional postnatal liver samples were obtained from Vitron (Tucson, AZ, USA). In addition, mRNA, DNA and microsomes isolated from livers acquired by XenoTech

LLC (Lenexa, KS, USA) were donated for use in this study. Available demographic data are summarized in Table 1. Tissues were stored at -70°C or below prior to preparation of subcellular fractions. To improve haplotype determination with the PHASE V2.1 algorithm (Stephens and Donnelly, 2003; Stephens et al., 2001) we added CYP2B6 genotyped DNAs from 33 liver tissue samples (sources as described under Liver Samples) and 37 DNA samples isolated from bone marrow transplant patients' blood for a total of 271 samples.

RNA Isolation

Total RNA was extracted from human liver tissues with a Qiagen RNeasy Mini kit (Valencia, CA, USA). An on-column DNase digest was performed. The quality of the total RNA was assessed by running a microfluidic Experion RNA StdSense chip (Bio-Rad Laboratories, Hercules, CA, USA). Only RNAs with an RNA quality index (RQI) of >6.5 were used for expression quantification. RQI values range from 1 to 10, 1 being totally degraded, 10 being the highest quality of RNA. The RNA quantity was determined spectrophotometrically by measuring an aliquot on a NanoDrop 1000 Instrument (Thermo Fisher Scientific, Rockford, IL, USA).

RNA Extraction and Quantitative Reverse Transcriptase qRT-PCR Analysis

Reverse Transcription Quantitative PCR. One step quantitative PCR reactions were performed for the house keeping gene cyclophilin A (*PPIA*) on 15 ng of total RNA per reaction using the qScript One-Step kit (Quanta BioSciences, Gaithersburg, MD, USA) and for the *CYP2B6* gene using the qScript One-Step SYBR kit (Quanta BioSciences,). Taq-Man reactions for PPIA (Applied Biosystems*)* were carried out on an Eppendorf Realplex instrument (Eppendorf, Hauppauge, NY, USA). Reverse transcription was performed for 3 minutes at 50°C and was immediately followed by a denaturation step at 95°C for 1 minute. Subsequently, 50 amplification cycles were performed consisting of denaturation at 95°C for 15 seconds and a combined annealing/extension at 60°C for 1 minute. SYBR green reactions for *CYP2B6* were carried out on an Applied Biosystems 7900 HT Real-Time PCR instrument (Life Technologies,

Carlsbad, CA, USA). A reverse transcription step of 10 minutes at 50°C was followed by an initial denaturation at 95°C for 5 minutes. Subsequently, 50 cycles of denaturation at 95°C for 15 seconds and a combined annealing/extension at 60°C for 45 seconds were performed. Serial dilutions of PCR amplicons were used to generate standard curves ranging in concentration from 10 to 10^7 molecules. Linear regression analysis was performed from the respective standard curves to calculate mRNA transcript numbers.

*Determination of Limit of Blank (LoB), Limit of Detection (LoD), and Limit of Quantification (LoQ).*In compliance with the MIQE guidelines, the level at which accurate quantification of real time PCR is attainable was determined using the following procedures with each gene. Known dilutions were made from cDNA ranging from three molecules/µl to 1500 molecules/µl (CYP2B6) or 1250 molecules/µl (PPIA). Real time PCR was carried out using these dilutions as input in triplicate reactions. The LoB, LoD and LoQ were determined as described by Armbruster et al. (Armbruster and Pry, 2008).

Assay Information:

CYP2B6: Equation: y=-3.6993048X+33.11937; R^2=0.999; E=0.863

Primer:

2B6F: 5' CAGCCACCAGAACCTCAACC (ex 6)

2B6R: 5' AAGGTCGGAAAATCTCTGAATCTCATA (ex 7)

Normalizer gene:

PPIA: Equation: y=-3.351X+35.61; R^2=0.999; E=0.99; Life Technology catalog #: 4333763

<u>Genomic DNA Isolation and Genotype Analyses</u>

Genomic DNA (gDNA) was isolated from ~25 mg of tissue using a DNeasy Tissue Kit (Qiagen, Valencia, CA, USA) or an Illustra Tissue and Cells genomic Prep kit (GE Healthcare, Piscataway, NJ, USA). gDNA was assessed by agarose gel electrophoresis for quality; concentration was measured using a NanoDrop 1000 spectrophotometer (Thermo Scientific, Waltham, MA). gDNA samples were diluted to 15 ng/µl.

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Genotyping was carried out on an Applied Biosystems 7900 HT Real-Time PCR System using predesigned Life Technology TaqMan assays for *CYP2B6* rs34223104 (-82T>C); rs3745274 (516G>T, Q172H), rs28399499 (983T>C, I328T) and rs3211371 (1459C>T R487C). Reactions were scaled to 8 µl and contained 4 µl KAPA PROBE FAST Universal 2X qPCR Master Mix (KAPA Biosystems, Woburn, MA, USA), 0.2 or 0.4 µl TaqMan assay mix, respectively, depending on whether it was a 20x or 40x mix, and 12-18 ng gDNA. Cycle conditions were slightly modified from those recommended by KAPA Biosystems and consisted of an initial denaturation for 3 minutes at 95°C, followed by 40 cycles of 95°C for 15 seconds and 60°C for 30 seconds. Each plate contained gDNA samples obtained from the Coriell Institute (Camden, NJ, USA) to serve as controls for known genotypes.

To detect *CYP2B6* rs2279343 (785A>G, K262R) a high resolution melting (HRM) curve assay was developed and performed as described (Twist et al., 2013). Briefly, a 70 bp long PCR product containing the SNP of interest was amplified from *CYP2B6* on the Illumina Eco Real-Time PCR System (Illumina, San Diego, CA, USA) and PCR products subjected to high resolution melt (HRM) curve analysis. HRM discriminated *CYP2B6* 785A>G as well as identified 777C>A SNP (rs45482602) that is located close-by and defines the *CYP2B6*3* allele. PHASE (version 2.1) software was used to construct haplotypes from *CYP2B6* genotype data as described (Stephens and Donnelly, 2003; Stephens et al., 2001). The n=201 samples of this study were phased with another 70 samples (CYP2B6 genotyped DNAs from 33 liver tissue samples (sources as described under Liver Samples) and 37 bone marrow transplant patients' blood DNAs) for a total of 271 samples to improve the accuracy of haplotype assignment.

TaqMan assays were also performed for the following sequence variations: *POR1*28 (*Cytochrome P450 Oxidoreductase, rs1057868), *AKR1D1* (Aldo-Keto Reductase family 1, member D1, rs1872930) and *PPARα* (Peroxisome Proliferator Activated Receptor *α*, rs4253728) using the modified conditions described above.

Preparation of Liver Microsomes

Human liver microsomes were prepared by differential centrifugation, essentially as described by Lu and Levin (Lu and Levin, 1972). Frozen liver samples were placed in homogenizing buffer (~3 mL/g liver; 50

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mM Tris.HCl, pH 7.4 at 4°C, containing 150 mM KCl and 2 mM EDTA) and allowed to thaw at 4°C. Liver samples were homogenized in Potter-Elvehjem-type glass mortars (round-bottom) with Teflon pestles utilizing a motor-driven tissue homogenizer (Caframo Model BDC-3030, Wiarton, ON, Canada). Nuclei and lysosomes were removed from the homogenate by centrifugation (~800 g_{max} for 15 min at 4°C). The resulting supernatant was subjected to further centrifugation (~12,000 g_{max} for 20 min at 4°C) and the supernatant fraction was subjected to ultra-centrifugation (~105,000 g_{max} for 70 min at 4°C). The resulting supernatant (cytosol) was stored at –70°C. The pellet (microsomal fraction) was removed from the centrifuge tube, transferred to a low-volume glass mortar, manually re-suspended in 0.25 M sucrose with a Teflon pestle and stored at -70ºC until use. Protein concentrations were determined with the Micro BCA Protein Assay kit (Pierce Chemical Co., Rockford, IL, USA) using bovine serum albumin (Sigma, St. Louis, MO, USA) as the standard.

Bupropion Hydroxylase Activity

Hydroxybupropion and bupropion were resolved by isocratic, reversed-phase HPLC based on a modification of the method described by Faucette et al. (Faucette et al., 2000). HPLC analyses were performed with a Hewlett Packard HP1100 HPLC system equipped with a programmable HP1100 series diode array detector (Hewlett Packard Instruments, Santa Clara, CA, USA). All data were collected and integrated with Agilent Chemstation 3D software, version B.03.07 (Santa Clara, CA, USA) set at 214 nm. Peaks of interest were separated on a Phenomenex (Torrance, CA, USA) Prodigy C_{18} column (4.6 mm X 15 cm, 3 µm particle size) preceded by a Phenomenex C_{18} guard column (4 X 3 mm i.d., 5 µm particle size). The mobile phase was a 79:21 mixture of 0.25% triethylamine containing 0.1% formic acid and acetonitrile and was delivered at a constant flow rate of 1 mL/min. The column temperature was maintained at 40°C. Under these conditions hydroxybupropion and bupropion had retention times of 4.8 and 8.6 min, respectively; total sample analysis time was 10.5 min. The column effluent was monitored at 214 nm. Hydroxybupropion was quantified by comparison of peak areas with those of analytical standards. The lower limit of quantification for hydroxybupropion was 0.06 µM. The analytical method was linear (r^2>0.998) over a standard concentration range of 0.06 to 200 µM. Standard curves run on 3

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separate occasions produced intraday CVs that ranged from 0.0 to 15.7%, whereas interday CVs ranged from 3.1 to 10.9%.

In vitro enzyme assays were performed in 96-well microtiter plates. Incubations (100 μL) contained human liver microsomes (30 μg of microsomal protein), potassium phosphate buffer (50 mM, pH 7.4), $MgCl_2$ (3 mM), EDTA (1 mM), and bupropion (500 μM) at the final concentrations listed. Reactions were initiated by the addition of an NADPH-generating system, consisting of NADP (1 mM), glucose-6-phosphate (5 mM), and glucose-6-phosphate dehydrogenase (1 U/mL), incubated at 37±0.1°C in a Thermo Forma (Marietta, OH, USA) Benchtop Orbital Shaker and terminated after 30 min by the addition of 50 μl of ice-cold acetonitrile. Protein was precipitated by centrifugation at 10,000 *g* for 10 min and a direct injection of an aliquot (70 μl) of the supernatant was analyzed by HPLC. Incubations were performed in triplicate. Bupropion stock solutions were dissolved in methanol, but the concentration of methanol present in incubation mixtures did not exceed 0.25%. The rate of bupropion hydroxylation was proportional to protein concentration and incubation time under the conditions used in these experiments.

Immunoquantitation of Microsomal CYP2B6 Protein

Human liver microsomes or insect cell microsomes containing recombinant human CYP2B6 were diluted with Bio-Rad XT Sample Buffer and XT Reducing Agent according to the manufacturer's instructions and heated for 5 min at 95°C. Microsomal proteins (1 to 40 μg per lane) were loaded on a polyacrylamide gel based on each sample's bupropion hydroxylase activity to insure that observed CYP2B6 protein levels would fall within the narrow, linear quantitative range available on Western immunoblot. Subsequently, microsomal proteins were separated on precast Criterion™ 26-well XT Bis-Tris 10% gels in Bio-Rad XT MOPS Running Buffer, pH 6.9, at 150 V for 2 hrs at 4°C. Proteins were transferred to nitrocellulose membranes using the Bio-Rad Criterion™ Blotter with Bio-Rad Tris/Glycine transfer buffer containing 39 mM glycine, 48 mM Tris, 0.0375% SDS and 20% methanol at 30 V for 2 hrs at 4°C. Membranes were blocked for 2 hrs at room temperature on a rocker in 10 mM Tris.HCl, 150 mM NaCl, 0.05% Tween-20, pH 8.0 containing 4% dry milk powder (M-TNT). Blocked membranes were placed in hybridization tubes (protein side facing inward), rotated horizontally over-night at 4°C and incubated with primary rabbit anti-

human CYP2B6 antibody that was diluted 1:5000-fold in M-TNT. Blots were washed with TNT (10 min) and subsequently washed with deionized water (10 min). The washes were repeated two additional times. Blots were then incubated with horseradish peroxidase-conjugated donkey anti-rabbit antibody diluted 1:40,000-fold in M-TNT at room temperature. After 2 hours, the blots were repeatedly washed with TNT followed by deionized water (5 min × 6) and then incubated with ECL Plus chemiluminescence reagents according to the manufacturer's instructions (GE Healthcare). Florescence was captured by exposing the blots to Hyperfilm ECL for 10 min. Films were scanned using a flatbed scanner and densitometric analysis of immunoreactive protein was performed using Kodak Molecular Imaging Software v. 4.0.4 (Eastman Kodak Co., Rochester, NY, USA). Microsomes from baculovirus-infected insect cells (SUPERSOMES^{TM}) expressing human CYP2B6 (BD Gentest) were used to derive a standard curve using 0, 5, 10, 33, 66 and 100 fmol of CYP2B6 protein. Pre-stained molecular weight markers served as negative control and a pooled, adult human liver microsomal sample was used as positive control. CYP2B6 standards and control samples were included on each membrane. Standard curves were linear over the range of standards and coefficients of determination (r^2 values) and ranged from 0.963 to 0.998. Values presented are the mean of two or more determinations.

Data and Statistical Analyses

Expression of mRNA transcripts was normalized for the expression of PPIA mRNA. Linear regression was used to determine the response range for standard curves comprised of recombinant cDNA-expressed CYP2B6 or hydroxybupropion that were then subsequently used to quantify CYP2B6 protein levels and rates of hydroxybupropion formation, respectively. Statistical analyses were performed using JMP version 10.0 (SAS, Cary, NC). CYP2B6 mRNA, protein and activity data were not normally distributed. Log transformation of CYP2B6 mRNA, protein and activity approximated normal sample distributions, but each failed the Shapiro-Wilk normality test (p<0.05). Spearman's rho correlation coefficients were calculated to measure associations between age and log-transformed mRNA, protein and activity data. Statistical analyses between age, gender, ethnicity and *CYP2B6* genotype with log-transformed CYP2B6 mRNA expression levels, protein contents, rates of bupropion hydroxylation, were

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compared with the Wilcoxon test or Kruskal-Wallis nonparametric ANOVA followed by a Dunn's post-hoc test. Log-transformed CYP2B6 RNA expression levels, protein contents and rates of hydroxybupropion formation were utilized in partitioning analyses to search for age-break points. Logworth values ≥ 1.95 and p values < 0.05 were considered significant. Groups were compared using Kruskal-Wallis nonparametric ANOVA followed by a Dunn's post-hoc test.

A linear regression model was developed in JMP (version 10) to assess the association of age, gender, ethnicity, *CYP2B6* genotype and exposure to known inducers on CYP2B6 activity or protein expression. The log of CYP2B6 activity or protein level was assigned as the dependent variable, and the other factors as independent co-variables. Following formulation of the model, each independent co-factor was removed from the model (one-at-a time) to assess the contribution of the factor to the association with CYP2B6 activity or protein level.

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Results

CYP2B6 mRNA expression, protein levels and bupropion hydroxylase activity

Microsomes and RNA were isolated from liver samples obtained from 24 fetal, 141 pediatric and 36 adult donors. mRNA preparations from 72 liver samples had RQI values <6.5, which was below the acceptance criteria established to generate meaningful quantitative mRNA expression data. Hence, quantitative mRNA expression data were obtained on only 129 of the 201 liver samples (Table 1). Although CYP2B6 mRNA expression was extremely low in fetal livers (Figure 1, Panel A), a dramatic increase in post-natal CYP2B6 mRNA expression was evident as early as the first day of life. Considerable variability in CYP2B6 mRNA expression was observed among the evaluated postnatal liver samples; mRNA expression levels varied >40,000-fold (range: 0.7 to 28744 molecules/reaction/15 ng total RNA).

Polyclonal antibodies raised against CYP2B6 revealed a predominant immunoreactive band with an apparent molecular mass of approximately 50 kDa in human liver microsomes. This immunoreactive protein co-migrated with full-length, cDNA-expressed CYP2B6. A representative immunoblot illustrating a range of protein contents is shown in Figure 2. Quantifiable levels of immunoreactive CYP2B6 protein were detected in all 201 microsomal preparations, although considerable inter-individual variation (316-fold, range: 0.15-47.39 pmol/mg protein) was observed in CYP2B6 protein content across the entire liver tissue panel (Figure 1, Panel B).

CYP2B6 activity was characterized by the rates that human liver microsomes catalyzed bupropion hydroxylation (results shown in Figure 1, Panel C). All 24 of the fetal liver microsomes failed to catalyze bupropion hydroxylation at discernable rates (*i.e.,* yielding concentrations above the limits of detection). Post-natal microsomes prepared from five of the pediatric and two of the adult donors also did not appear to catalyze bupropion hydroxylation. An additional 21 pediatric and two adult microsomal preparations formed small quantities of hydroxbupropion, but at levels below the limit of quantitation of the assay. CYP2B6 activity appears as early as the first day of life; bupropion hydroxylase activity was measurable (15.8 pmol/mg/min) in liver microsomes prepared from a 0 day-old donor. Consistent with the inter-individual variation noted in mRNA and protein expression levels, considerable inter-individual variation was observed in post-natal CYP2B6 activity where bupropion hydroxylase activity varied 648-fold (range: 5.73-3714 pmol/mg protein/min) among the panel of liver microsomes.

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CYP2B6 mRNA, protein and activity data were not normally distributed. Log transformation of CYP2B6 mRNA, protein and activity approximated normal sample distributions, but each failed the Shapiro-Wilk normality test ($p < 0.05$). Hence, relationships between various parameters (*e.g.,* age, gender, etc.) with log-transformed mRNA, protein and activity data were subsequently evaluated using non-parametric tests. Significant associations between age and log-transformed mRNA expression (Spearman's $\rho = 0.268$, $p < 0.002$), protein content ($\rho = 0.211$, $p < 0.003$) and bupropion hydroxylase activity ($\rho = 0.227$, $p < 0.006$) were observed. Log-transformed mRNA expression was significantly associated with log-transformed CYP2B6 protein levels ($\rho = 0.293$, $p < 0.001$) and log-transformed bupropion hydroxylase activity ($\rho = 0.560$, $p < 0.0001$). Log-transformed CYP2B6 protein levels were also significantly associated with log-transformed bupropion hydroxylase activities ($\rho = 0.828$, $p < 0.0001$).

To determine if any developmental thresholds in CYP2B6 expression or activity were present, partitioning analyses were performed on log-transformed data. A break-point was identified for log-transformed mRNA expression at approximately 0.1 years of age (logworth value 71.6), which corresponds to the end of the neonatal period. Samples from donors with an age greater than or equal to 0.1 yrs of age had mean expression levels (geometric mean = 721 molecules/15 ng total RNA) 1000-fold greater than those from younger pre-natal and neonatal donors (geometric mean = 0.72 molecules/15 ng total RNA). Similarly, break points were observed for log-transformed protein levels at 0, 2 and 11.5 and 39 years of age (logworth value scores of 8.54, 2.04, 2.11 and 5.30, respectively). Mean log-transformed CYP2B6 protein levels were ~2-fold higher in fetal samples (geometric mean = 2.19 pmol/mg protein) than samples from donors between the ages of 0 and 2 years (geometric mean = 0.85 pmol/mg protein). Protein levels subsequently increased in the samples from donors between the ages of 2 and 11.5 years of age (geometric mean =2.39 pmol/mg protein), followed by a return to protein levels similar to those observed in the 0-2 age group in samples from donors between 11.5 and 39 years of age (geometric mean = 1.06 pmol/mg protein), with a subsequent rise in protein levels in samples from donors over 39 years of age (geometric mean = 4.62 pmol/mg protein). Break points in log-transformed CYP2B6 activity appeared at 11 months, 12.4 and 39 years of age (logworth values of 3.52, 5.25 and 1.98, respectively). The geometric means of log-transformed bupropion hydroxylase activities were 19.8 pmol/mg protein/min in samples from pediatric donors less than 11 months of age, 129 pmol/mg protein/min in samples from

donors 11 months to 12.4 years of age and 36 pmol/mg protein/min in samples from donors between 12.4 and 39 years of age, and 246 pmol/mg protein/min in samples from donors over 39 years of age.

Based on a composite of the breakpoints identified above, the following age groups were constructed to uniformly evaluate changes in log-transformed CYB2B6 mRNA expression, protein content and bupropion hydroxylase activity: fetal (prior to birth), 0 to <1, 1 to <12, 12 to <18 and >18 years of age and the results are shown in Table 2. Similar to the results presented above, fetal mRNA expression was significantly different from all other age groups (Kruskal-Wallis followed by Dunn, $p < 0.0005$), but all other age groups were not statistically different. In contrast, fetal CYP2B6 protein content was not significantly different from protein levels observed in samples from donors aged 1 to <12 and >18 years of age. Significant decreases (> 2-fold) were observed between CYP2B6 protein content in the 0 to <1 and 12 to <18 years of age groups and the aforementioned groups (Kruskal-Wallis followed by Dunn $p < 0.005$ and $p < 0.03$, respectively), although these latter two groups were not significantly different from one another. Rates of CYP2B6 activity (as measured by rates of bupropion hydroxylation) among the age groups segregated similar to that observed for protein, with the exception that activity in the fetal samples (no activity observed) was significantly different compared with rates from all other groups ($p < 0.0001$). Rates of CYP2B6 activity in the 0 to <1 year of age group were significantly lower (> 5-fold) than those of either the 1 to <12 and >18 years of age groups ($p<0.0007$), as were the CYP2B6 activity rates of the 12 to <18 years of age group ($p< 0.03$). No other significant differences between age groups were observed for CYP2B6 activity.

CYP2B6 expression and activities were compared among African American (n = 51), Caucasian (n = 92) and Hispanic (n = 6) samples along with a group combining samples with no reported ethnicity (n = 50) (Note: Two individuals were excluded from the comparison, one identified as a Native American, the other as a Pacific Islander). No significant differences in CYP2B6 mRNA expression, protein levels or activities were observed among each of the ethnic groups. Log-transformed CYP2B6 mRNA expression, protein levels and activities did not differ significantly between females and males either (Wilcoxon, $p = 0.765$, 0.180 and 0.071, respectively), although mean CYP2B6 protein levels and activities were slightly higher

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in females compared to males (geometric mean, female/male: 1.96 versus 1.50 pmol CYP2B6/ mg protein; 104 versus 67 pmol/mg/min, respectively).

Donor sample quality and the potential for degradation of mRNA and enzymatic protein between the time of death and tissue freezing, known as post-mortem interval or PMI, is always a concern with studies of the type described here. PMI was documented for 118 samples and ranged from 25 minutes to 25 hours (mean ± S.D. 9.7±5.9 hrs). PMIs were not significantly correlated with log-transformed CYP2B6 mRNA expression (ANOVA, p = 0.714; data not shown) nor protein levels (p = 0.234), but were significantly correlated with bupropion hydroxylase activities (p = 0.007). However, the line of best fit between PMI and the rates of bupropion hydroxylation had a positive slope, indicating that bupropion hydroxylase activities increased with increasing PMIs, a situation contrary to expectation. Because there are differences in the procedures used to process organs for research, we separated data from tissues recovered and frozen (but not perfused) from data arising from tissues which were perfused with preservation solution with the potential for transplantation, and subsequently evaluated the relationship between PMI and CYP2B6 mRNA expression, protein levels and activities. Although perfused, post-natal livers tended to have a longer PMI (mean ± S.D.: 15.3±5.8 hrs) than samples from non-perfused livers (mean ± S.D.: 8.2±4.8 hrs), PMIs from neither group were significantly correlated with CYP2B6 mRNA expression (ANOVA, p = 0.475 and 0.610, respectively), protein levels (p = 0.554 and 0.472, respectively) or bupropion hydroxylase activities (p = 0.772 and 0.934, respectively). Because there was no detectable bupropion hydroxylase activity observed in the fetal liver microsomes used in this study, it is important to note that these same microsomal samples have demonstrated CYP3A7 enzymatic activity, catalyzing the 2α- and 16β-hydroxylation of testosterone and the 16α-hydroxylation of DHEA (Leeder et al., 2005).

It should also be noted that liver samples were collected on a convenience basis. As such, no power analyses were conducted prior to study initiation to determine the minimum number of samples required to detect statistically significant differences in each of the various groupings. Thus, there is the potential for false negative associations in groups containing small sample numbers, such as those in the neonatal age range and for certain genotypes.

Genotype analyses

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Seven *CYP2B6* sequence variations were interrogated and haplotypes constructed. Table 3 provides a summary of the SNPs present in each haplotype and their respective CYP nomenclature designations as well as estimated allele frequencies. For reference and ease of comparison, all allelic variants defined to date are listed in Table 3. None of the tentative novel haplotypes (**N1* through **N6*) corresponded to haplotypes defined by the Cytochrome P450 Nomenclature Committee.

The samples from this study were analyzed in combination with an additional 70 samples to increase the accuracy of the PHASE output data. The three most common allelic variants were *CYP2B6*5, *6* and **18* with frequencies of 9.03%, 27.55% and 3.23%, respectively. SNP frequencies were in Hardy Weinberg Equilibrium, with the exception of 983 T>C (rs28399499), which is part of the *CYP2B6*16* and **18* haplotypes. This is likely due to the presence of two homozygous *CYP2B6*18/*18* subjects among the liver tissue samples.

As shown in Table 4, diplotypes could not unequivocally be determined for 11 tissue samples. Different haplotype combinations may theoretically exist in these samples based on the genotype data for seven SNPs obtained from a total number of 271 samples including the 201 of the liver tissue panel. These samples have more likely and less likely (or 'alternate') diplotype assignments as listed in Table 4.

Frequencies of *POR* (rs1057868), *AKR1D1* (rs1872930) and *PPARα* (rs4253728) variant alleles were calculated to be 27.61%, 23.13% and 21.14%, respectively, in the liver tissue panel. These were in Hardy Weinberg Equilibrium and within ranges expected for a predominantly Caucasian sample cohort.

Impact of genetic variation on CYP2B6 mRNA expression, protein levels and activity

Due to the differences in mRNA expression and bupropion hydroxylase activity noted between pre-natal and post-natal samples, the impact of genetic variation on CYP2B6 mRNA expression, protein levels and activity was investigated only with post-natal samples and the results are shown in Figure 3. Compared with expression of the reference sequence, significantly lower mRNA expression (~ 7-fold decrease, Kruskal-Wallis followed by Dunn, p = 0.038) was observed in samples from donors homozygous for the SNP at position 516 G>T (rs3745274) which is part of the *CYP2B6*6* haplotype. There was also a trend for lower mRNA expression (~7-fold reduction) in donor samples that were homozygous for 785 A>G

(rs2279343), which is found in several allelic variants, including the *CYP2B6*6* haplotype (Table 3). However, this decrease was not statistically significant (Kruskal-Wallis followed by Dunn, p = 0.073). Neither of these SNPs was associated with significant changes in either protein content or bupropion hydroxylase activity, nor was any other SNP found to be associated with significant differences in mRNA expression, protein levels or CYP2B6 activity.

Although several *CYP2B6* diplotypes (consisting of phased haplotypes) had higher or lower mean RNA expression, protein content or bupropion hydroxylase activity (Figure 3) compared with the values for samples from *CYP2B6*1/*1* donors, no significant associations between *CYP2B6* diplotypes and CYP2B6 mRNA expression, protein levels or bupropion hydroxylase activities were observed (Tukey-Kramer). There were also no associations between CYP2B6 mRNA expression, protein levels or CYP2B6 activity with either *POR1*28* (rs1057868), *AKR1D1* (rs1872930) or *PPARα* (rs4253728).

Modeling of CYP2B6 activity and protein levels

A linear regression model incorporating age, gender, ethnicity, *CYP2B6* diplotype and known exposure to CYP2B6 chemical inducers was formulated and the impact of each of the factors on log-transformed CYP2B6 activity or protein levels was assessed. The model accounted for 26% and 23% of the variability in CYP2B6 activity and protein expression, respectively. Individual contributions of each of the factors to CYP2B6 activity and protein expression were as follows: age, 5.1% and 6.3%; gender, 1.5% and 1.0%; ethnicity, 9.6% and 5.9%; genotype, 6.4% and 7.3%; and known inducer exposure, 3.0% and 2.6%, respectively. The low contribution of known inducer exposure to the model may be an underestimate of the true impact of CYP2B6 inducers on inter-individual variability of CYP2B6 activity and protein levels. For most of the tissue donors included in this study, medication and drug exposure history were not well documented; only seven individuals had a documented history of chronic alcohol use or exposure to medications known to cause CYP2B6 induction. Hence, there is a strong possibility that there are donors in our sample set that may have had exposure to inducing agents that are not identified as such.

Discussion

Information regarding the effects of development on CYP2B6 expression and activity are scarce. Two early studies failed to detect CYP2B6 mRNA or protein expression in fetal liver samples (Hakkola et al., 1994; Maenpaa et al., 1993); A few years later, Tateishi et al. (Tateishi et al., 1997) compared liver microsomal cytochrome CYP protein expression between one group of ten subjects less than one year of age (6 infants and 4 perinatal subjects) with a second group of ten subjects over the age of one year (including 6 pediatric aged subjects (2-16 yrs) and 4 adults (22-72 yrs) and found higher levels of CYP2B6 expressed after infancy than during infancy. CYP2B6 protein was detected in only 2 of the 10 samples from donors between 37 weeks gestation and 10 months of age (2.7 ± 5.9 pmol/mg protein) versus 7 out of 10 samples for those older than 10 months of age (19.4 ± 23.9 pmol/mg protein).

The most comprehensive study of CYP2B6 developmental changes to date, characterized CYP2B6 protein levels in 217 fetal and pediatric liver samples from donors ranging from 10 weeks gestation to 17 years of age and compared protein expression levels with a limited genotype analysis (only *CYP2B6*6* was tested) (Croom et al., 2009). Samples from donors with a postnatal age >30 days had 2-fold higher CYP2B6 levels (median = 1.3; range = 0.0 to 23.9 pmol/mg protein) than samples from donors younger than 30 days of age (median = 0.6; range = 0.0 to 36.7 pmol/mg protein). CYP2B6 protein was detected in 64% of the fetal samples, whereas 90% of the samples from donors over the age of 6 months expressed quantifiable protein levels. It should be noted that none of the aforementioned studies characterized CYP2B6 activity in their samples. Several other studies have found no difference in CYP2B6 expression or activity in relation to age, but each of these studies included only a few pediatric microsomal samples between the ages of 2 and 18 in their sample set (Desta et al., 2007; Hesse et al., 2004; Hofmann et al., 2008; Parkinson et al., 2004).

In the current study, we detected CYP2B6 protein in all of the microsomes prepared from liver samples obtained from 201 donors that spanned a wide range of ages (~11 weeks gestation to 79 years of age). Our limit of quantitation was 5 fmol/lane, which represents a six-fold reduction over the limit of quantitation reported by Croom et al. (31 fmol/lane) (Croom et al., 2009) and is comparable to limits of

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quantitation determined by highly accurate HPLC/MS/MS methods (Groer et al., 2014) This increased sensitivity potentially explains, in part, our success in detecting CYP2B6 in all of our liver samples.

Although infant and post-infant levels of CYP2B6 protein in our study were similar to those noted in previous studies (Croom et al., 2009; Hesse et al., 2004; Hofmann et al., 2008; Tateishi et al., 1997), we observed elevated levels of fetal CYP2B6 (~4-fold). Interestingly, fetal livers contained extremely low levels of CYP2B6 mRNA relative to post-natal samples and all of the fetal microsomal samples failed to catalyze bupropion hydroxylation. It is unclear whether the fetal protein observed in our samples is an inactive form of CYP2B6, a previously unrecognized CYP2B isoform or another protein of similar size that possesses an epitope that is recognized by the CYP2B6 antibody. Whatever the case, the protein recognized by the CYP2B6 antibody appears to have little or no capacity to catalyze bupropion hydroxylation and merits further investigation.

Our results demonstrate that CYP2B6 activity can appear as early as the first day of life. Liver microsomes prepared from a donor that died on their day of birth had measurable CYP2B6 activity (15.8 pmol/mg protein/min) and expression of mRNA (108 molecules/15 ng total RNA) that vastly exceeded those observed in the fetal samples. Our data suggest that CYP2B6 increases through infancy and by 1 year of age, CYP2B6 levels and activity may approach those of adults, which are 2- to 5-fold higher than levels/activities in infants. We observed a decline in CYP2B6 protein and activity during adolescence followed by a return to child/preadolescent levels in adulthood. However, it is possible that the decrease observed during adolescence is an artifact of our sample set, as this trend has not been corroborated by other studies (Croom et al., 2009; Hesse et al., 2004; Hofmann et al., 2008; Parkinson et al., 2004).

Tateishi et al. originally proposed that CYP developmental trajectories followed one of three paths (Tateishi et al., 1997). This model was based on only 20 human liver microsomal samples, but was corroborated in studies by Hines that provided supporting evidence for this classification for several hepatic CYP enzymes utilizing much larger sample numbers (Hines, 2007; 2013). The first group consists of those enzymes that are expressed at the highest levels in the fetus with a subsequent decline in expression following birth; CYP3A7 is a member of this group of enzymes. The second group consists of enzymes that are expressed in the fetus and remain at a relatively constant level throughout post-natal

development; CYP2C19 exemplifies the members of this group. The third group of enzymes is expressed at very low levels (or not at all) in the fetus and just after birth, but demonstrates marked increases in expression thereafter. CYP3A4 and CYP2D6 belong in this last group of enzymes. Based on the protein expression observed by Croom et al. (Croom et al., 2009), Hines classified CYP2B6 as a group 2 enzyme (Hines, 2013). However, the results of the current study suggest that CYP2B6 is perhaps more accurately classified as a group 3 enzyme, based on the low level of mRNA expression and lack of bupropion hydroxylase activity in fetal liver followed by a subsequent rise in expression of CYP2B6 mRNA and protein and in catalytic activity through the first year of life.

We observed considerable inter-individual variation in CYP2B6 mRNA expression, protein levels and activity across our entire set of post-natal human liver microsomes (mRNA, ~40,000-fold; protein, ~300-fold; activity, ~600-fold). However, the geometric mean of log-transformed CYP2B6 mRNA expression, protein levels and activity in infants were only 2- to 5-fold lower than those of subjects older than 1 year of age. Inter-individual variability in CYP2B6 expression and activity was not significantly associated with differences in gender, ethnicity nor with *CYP2B6* genetic variation. Although several *CYP2B6* diplotypes were noted to have higher or lower mean expression or activity, these were not significant. Even individuals homozygous for the *CYP2B6*6* allele, which causes reduced protein expression (Hofmann et al., 2008; Lang et al., 2001) and has been linked with lower bupropion hydroxylase activity both in vitro and in vivo (Benowitz et al., 2013; Desta et al., 2007; Hofmann et al., 2008) did not have significantly different expression levels or bupropion hydroxylase activities from those individuals homozygous for the *CYP2B6*1* wild type allele. Similar to our findings, Croom et al. (Croom et al., 2009) also did not observe any significant difference in protein expression levels between samples genotyped as *CYP2B6*6/*6* and those not carrying this allele.

No significant associations were also observed for ethnicity or gender, although females had mean CYP2B6 protein levels and activities that were slightly higher than those found in males. Gender differences in CYP2B6 are controversial. Although a few studies have reported gender differences in CYP2B6 protein or activity (Al Koudsi and Tyndale, 2010; Lamba et al., 2003; Naidoo et al., 2014), a number of other studies have not observed this phenomenon (Croom et al., 2009; Hesse et al., 2004;

Hofmann et al., 2008; Parkinson et al., 2004). Exposure to inducers potentially could account for a portion of the large variability observed in this study, but only 2 individuals had known exposure to drugs capable of inducing CYP2B6 (both 7-year olds) and 5 individuals had a history of chronic alcohol use (5 adults), which has been shown to cause CYP2B6 induction (Ferguson et al., 2013; Hesse et al., 2004). However, it should also be noted that the medication and drug exposure history was not well documented for most of the tissue donors.

Individualizing doses of CYP2B6 medications to achieve therapeutic concentrations may prove to be challenging due to the polymorphic nature of the *CYP2B6* gene and the fact that *CYP2B6* is highly inducible. Further complicating the predictive ability of phenotype from genotype is that for some CYP2B6 variants, the relative catalytic activity appears to be dependent on the substrate examined (Helsby and Tingle, 2011a; Helsby et al., 2010; Helsby and Tingle, 2011b). For example, individuals with a *CYP2B6*6* allele demonstrate decreased efavirenz 8-hydroxylation and clearance *in vivo (Naidoo et al., 2014)*, but increased cyclophosphamide 4-hydroxylation and clearance (relative to *CYP2B6*1* homozygotes) (Xie et al., 2006). Hence, in order to accurately estimate CYP2B6 biotransformation capacity for a particular medication in an individual, an understanding of the influence of CYP2B6 genetic variation on the clearance of the drug will be required, and it may even be necessary to empirically determine drug clearance for some compounds, given the wide range of CYP2B6 inter-individual variability.

In conclusion, this study has demonstrated that CYP2B6 mRNA expression levels and CYP2B6 activity are extremely low during fetal development, increase after birth, and by 1 year of age, approach levels and activity observed in adults. Although a protein reacted with antibodies raised against CYP2B6 in fetal liver microsomes, this protein had no activity. Hence, it is unclear whether the protein observed in fetal samples is truly CYP2B6. CYP2B6 protein was observed in neonatal samples (confirmed by the presence of bupropion hydroxylase activity), increased to adult levels somewhere between 1 and 2 years of age, and possibly declined slightly during adolescence. Considerable inter-individual variation in CYP2B6 mRNA expression, protein levels and activity was observed in our extensive cohort of post-natal human liver microsomes (mRNA, ~40,000-fold; protein, ~300-fold; activity, ~600-fold). The extremely wide range of inter-individual variability in CYP2B6 expression and activity in the present study was significantly

associated with age following log transformation of the data. Surprisingly, CYP2B6 inter-individual variability was not significantly associated with genetic variation in *CYP2B6,* nor with differences in gender or ethnicity, suggesting that factors other than these are largely responsible for the wide range of variability in CYP2B6 expression and activity observed among a large group of individuals/samples.

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Authorship contributions

Participated in research design: A Gaedigk, R Gaedigk, Leeder, Pearce

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Contributed new reagents or analytic tools: n/a

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Wrote or contributed to the writing of the manuscript: A Gaedigk, R Gaedigk, Leeder, Pearce, Riffel, Twist

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Footnotes

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Legends for Figures

Figure 1. Relationship between age and CYP2B6 mRNA transcript levels (A), protein expression (B) and activity (C) in human liver samples and microsomes.

CYP2B6 mRNA transcript levels were determined by qRT-PCR, protein levels by western immunoblot and bupropion hydroxylase activity by HPLC/UV, as described under Methods. The x-axis was expanded for the pre-natal and 0 to 2 year age range to better visualize the data in this range (grey-filled circles). Note that no activity was detected in fetal liver microsomes, explaining the absence of respective data points in (C). CYP2B6 protein determinations were performed in duplicate, whereas CYP2B6 mRNA transcript determinations and bupropion hydroxylase activity determinations were performed in triplicate.

Figure 2. Immunoquantitation of CYP2B6 protein by western blot analysis.

A representative western immunoblot showing the relative amounts of CYP2B6 protein from 1-10 µg of microsomal protein in a random set of human liver microsomes. Immunoreactive CYP2B6 content was determined using a rabbit anti-human CYP2B6 antibody (Corning Gentest). A five point standard curve from 5 to 100 fmol of heterologously expressed CYP2B6 (Corning Gentest; rCYP2B6, [a]lot 5 and [b]lot 18 as indicated) was used to quantify CYP2B6 protein. Pooled, adult human liver microsomes (HLM) served as a positive control, as well as an inter-assay control, and a molecular weight marker (MWM) was included to show protein size and also served as a negative control. Microsomal protein was loaded in proportion to bupropion hydroxylase activity to insure that CYP2B6 protein levels would fall within the linear, quantitative range of the standard curve. CYP2B6 protein determinations were performed in duplicate for each microsomal sample.

Figure 3. Relationship between diplotype and CYP2B6 mRNA transcript levels (A), CYP2B6 protein content (B) and bupropion hydroxylase activity (C) in human liver samples and microsomes.

Solid diamonds represent post-natal samples and open circles represent fetal samples. Boxes represent the interquartile values and the line within each box represents the median value for post-natal samples. Whiskers represent boundaries for outliers (1.5 times interquartile boundaries). Note that no activity was

detected in fetal liver microsomes, explaining the absence of respective data points in (C). CYP2B6 protein determinations were performed in duplicate, whereas CYP2B6 mRNA transcript determinations and bupropion hydroxylase activity determinations were performed in triplicate.

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Tables

Table 1 Demographic data for liver tissue samples

	Prenatal with RNA	Pediatric w/o RNA	Pediatric with RNA	Adult w/o RNA	Adult with RNA
Tissue Source					
Seattle	14	0	0	0	0
UMB	10	48	20	8	2
LTCDS (Minnesota)	0	1	33	0	0
LTCDS (Pittsburgh)	0	2	13	0	24
Xenotech	0	8	13	1	0
Vitron	0	3	0	1	0
Total n=201	**24**	**62**	**79**	**10**	**26**
RNA Quality					
RNA RQI (range)	8.8-10	0-6.4	6.5-9.8	0-4.4	7.4-9.9
Post Mortem Interval					
Range (hrs)	1-3	3-24	0.4-25	2-16	4-6
Median (hrs) (not perfused; perfused)	2 (2, NA)	10 (9, 19)	11 (6, 14)	6 (5, 10)	5 (5, NA)
Total n=118 (perfused)	9 (0)	59 (8)	39 (13)	9 (1)	2 (0)
Demographics					
Age [range]	78-147 (PCA, days)	.01 – 17.5 yrs	0 – 17 yrs	18 – 47 yrs	23-79 yrs
Caucasian	4	32	37	7	12
African American	6	23	7	3	12
Hispanic	0	3	1	0	2
Other [a]	14	4	34	0	0
Gender (male/female) [b]	9/10	40/21	49/29	8/2	13/13

NA: Not applicable

[a] Other include Pacific islander, Native American, and those for which ethnicity is unknown

[b] Gender was unknown for 5 pre-natal and 2 pediatric samples (one with and one without RNA).

Table 2 CYP2B6 mRNA expression, protein content and bupropion hydroxylase activity

	Prenatal	**Pediatric**			**Adult**
	78-147 (PCA, days)	0-<1 years	1-<12 years	12 -<18 years	18 - 79 years
mRNA expression (molecules/15ng) Arithmetic Mean Geometric Mean	 0.72 0.58	 599 274	 4503 961	 673 283	 3364 700
mRNA expression Range	0.22-3.4	0.70-1479	2.29-28,744	16.8-2751	4.6-17,170
mRNA expression Fold-variation	16	2113	12551	164	3700
Sample number	24	12	44	23	26
Protein expression (pmol/mg protein) Arimthetic Mean Geometric Mean	 2.35 2.19	 1.23 0.86	 5.11 2.18	 1.35 1.02	 7.65 2.93
Protein expression Range	0.73-3.55	0.24-9.04	0.15-47.39	0.2-9.59	0.30-32.00
Protein expression Fold-variation	5	38	316	48	107
Sample number	24	28	64	49	36
Activity (pmol/mg protein/min) Arithmetic Mean Geometric Mean	 0 0	 55 24	 347 125	 88 43	 523 143
Activity Range [a]	n/a	5.74-553	6.34-3714	5.73-508	5.95-2366
Activity Fold-variation	n/a	96	585	88.7	398
Sample number	24	19	58	37	32

[a]CYP2B6 activity range covers lowest to highest measurable activities for each age group.

Table 3 *CYP2B6* haplotype definitions and allele frequencies

CYP2B6	rs34223104 -82 T>C	rs3745274 516 G>T Q172H	rs34646544 769 G>A D257N	rs45482602 777 C>A S259R	rs2279343 785 A>G K262R	rs28399499 983 T>C I328T	rs3211371 1459 C>T R487C	Allele frequency (%)
**1*	T	G	G	C	A	T	C	56.45
**3*	T	G	G	**A**	A	T	C	0.11
**4*	T	G	G	C	**G**	T	C	1.64
**5*	T	G	G	C	A	T	**T**	9.03
**6*	T	**T**	G	C	**G**	T	C	27.55
**7*	T	**T**	G	C	**G**	T	**T**	>0.001
**8*	T	G	G	C	A	T	C	0
**9*	T	**T**	G	C	A	T	C	0.45
**10*	T	G	G	C	A	T	C	0
**11*	T	G	G	C	A	T	C	0
**12*	T	G	G	C	A	T	C	0
**13*	T	**T**	G	C	**G**	T	C	0
**14*	T	G	G	C	A	T	C	0
**15*	T	G	G	C	A	T	C	0
**16*	T	G	G	C	**G**	T	**T**	>0.001
**17*	T	G	G	C	A	T	C	0
**18*	T	G	G	C	A	**C**	C	3.23
**19*	T	**T**	G	C	**G**	T	C	0
**20*	T	**T**	G	C	**G**	T	C	0
**21*	T	G	G	C	A	T	C	0
**22*	**C**	G	G	C	A	T	C	1.15
**23*	T	G	G	C	A	T	C	0
**24*	T	G	G	C	A	T	C	0
**25*	T	G	G	C	A	T	**T**	0
**26*	T	**T**	G	C	**G**	T	C	0
**27*	T	G	G	C	A	T	C	0
**28*	T	G	G	C	A	T	C	0
**33*	T	G	G	C	A	T	**A**	0
**34*	T	**T**	G	C	**G**	T	**A**	0
**35*	T	G	G	C	A	T	C	0
**36*	T	**T**	G	C	**G**	T	C	0
**37*	T	**T**	G	C	**G**	T	C	0
**38*	T	**T**	G	C	**G**	T	C	0
**N1*	T	**T**	**A**	C	A	T	C	0.10
**N2*	**C**	G	G	**A**	A	T	C	0.08
**N3*	T	**T**	**A**	C	**G**	T	C	0.09
**N4*	**C**	G	G	C	A	**C**	C	0.06
**N5*	T	**T**	G	C	**G**	**C**	C	>0.001
**N6*	T	G	G	C	**G**	**C**	C	>0.001

Table 3 (continued)

Nucleotide numbering is based on the cDNA NCBI Reference Sequence NM_000767.4.

Polymorphic nucleotides are shown in bold. Twelve variants are shown in gray; these are not identified by this SNP panel and if present, are defaulted to a *CYP2B6*1* assignment. *CYP2B6*29* and **30* are not listed because these are *CYP2B7/CYP2B6* and *CYP2B6/CYP2B7* hybrids, respectively, and *CYP2B6*31* and **32* have not yet been released. The novel haplotypes identified in this study are labeled **N1* through **N6*.

Allele frequencies are estimates based on PHASE V2.1 analysis that comprised a total of n=271 samples including the n=201+33 samples of the liver tissue panel.

Table 4 *CYP2B6* Diplotype Frequencies

CYP2B6 diplotype	Alternate diplotype (probability)	Number of subjects (n=201)	Genotype frequency (n=201)
**1/*1*		58	28.86
**1/*4*		5	2.49
**1/*5*		24	11.94
**1/*6*		74	36.82
**1/*18*		4	1.99
**1/*22*		2	1.00
**4/*6*		1	0.50
**5/*5*		2	1.00
**5/*6*		6	2.99
**6/*6*		12	5.97
**18/*18*		2	1.00
**4/*5*	**1/*16* (0.01)	1	0.50
**6/*N1*	**9/*N3* (0.48)	1	0.50
**3/*22*	**1/*N2* (0.5)	1	0.50
**18/*22*	**1/*N4* (0.14)	2	1.00
**6/*18*	**1/*N5* (0.01)	5	2.49
**4/*18*	**1/*N6* (0.02)	1	0.50

CYP2B6 haplotypes were determined by PHASE V2.1 and haplotypes assigned according to *CYP2B6* nomenclature and summarized in Table 3. For 11 of the 201 liver tissue samples, haplotype combinations could not unequivocally be determined, i.e. there is a probability that a subject has an alternate diplotype. The less likely diplotype is provided as the 'alternate diplotype'; probabilities are shown in brackets.

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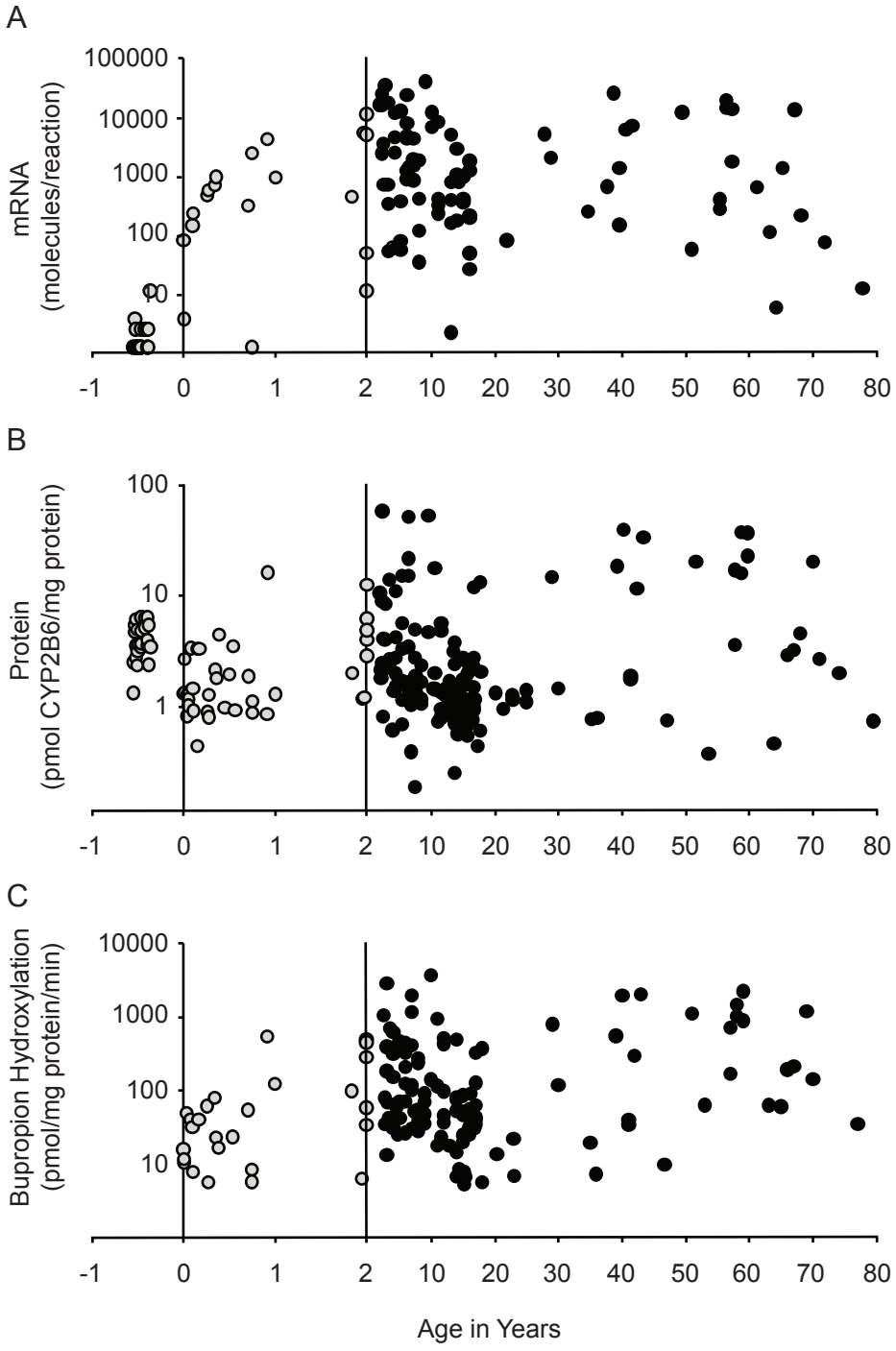

Figure 1

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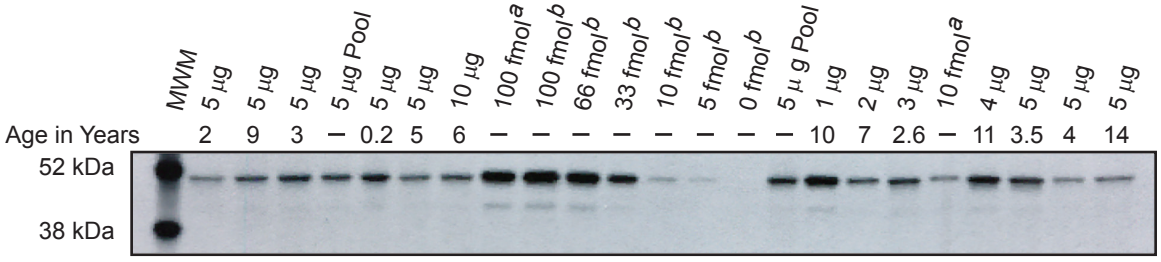

Figure 2

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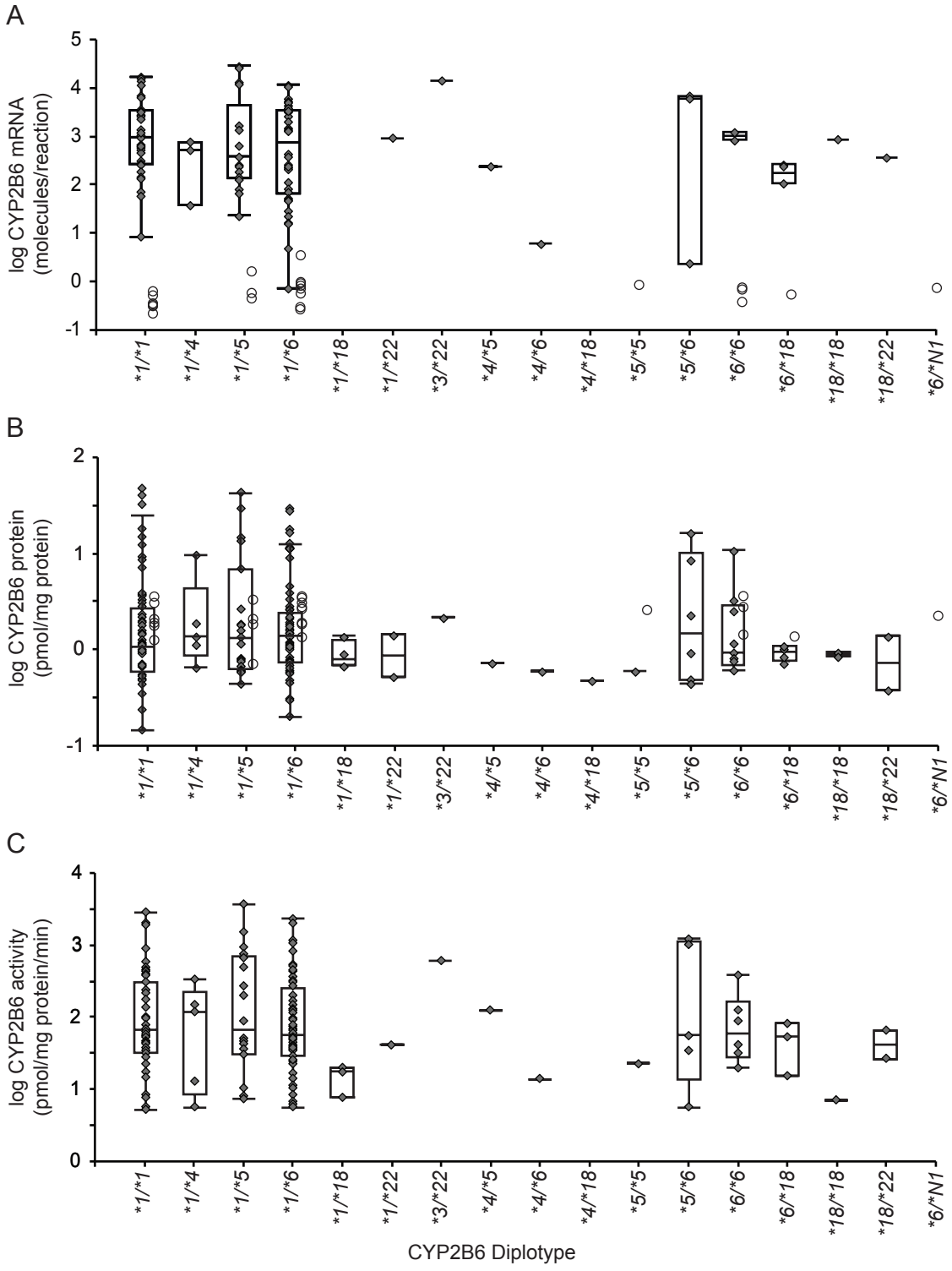

Figure 3