ABSTRACT

CROOM, EDWARD LEE. Human Hepatic Expression of CYP2B6: Developmental Pattern and In Vitro Bioactivation of Chlorpyrifos. (Under the direction of Dr. Ernest Hodgson and the late Dr. Randy L. Rose).

Chlorpyrifos is a widely used organophosphorothioate insecticide. Known human exposures to chlorpyrifos range from low dietary levels to the intentional ingestion of concentrated chlorpyrifos solutions as a means of suicide. Chlorpyrifos derives its in vivo toxicity through bioactivation by the cytochromes P450 (CYPs) to the neurotoxic metabolite, chlorpyrifos-oxon (CPO). Chlorpyrifos-induced toxicity occurs when the level of CPO produced exceeds the capacity to detoxify CPO before acetylcholinesterase inhibition occurs. Several human esterases detoxify CPO, the most efficient being the serum esterase, paraoxonase 1 (PON1). The capacity of PON1 to detoxify CPO is understood to depend on genotype, age and diet. PON1 expression can be developmentally delayed not plateauing until at least six months of age.

The ability to produce CPO has not been as well studied in humans and there are questions regarding the impact of age, genetic variation on chlorpyrifos bioactivation. To assess CPO production variability, a series of in vitro metabolism studies were conducted with individual human liver microsomes incubated with chlorpyrifos. CPO production varied over 14-fold and was predicted by CYP2B6 activity but no relationship with CP2B6 genotype was observed.

CYP expression can change substantially during development. However, CYP2B6 ontogeny has been poorly characterized. Earlier reports suggested a general lack of CYP2B6 expression in neonatal and fetal liver samples. CYP2B6 levels in 220 individual human liver microsomal samples ranging from 10 weeks gestation to 17 years were semi-quantitatively

measured by western blot. CYP2B6 protein expression was determined to be significantly higher after the neonatal period and the percent of samples with detectable CYP2B6 protein increased from a low of 60% detectable in the first-trimester to over 90% detectable in samples from donors over 11 years of age. CYP2B6 is highly efficient at producing CPO,

Human Hepatic Expression of CYP2B6: Developmental Pattern and In Vitro Bioactivation of Chlorpyrifos.

by
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DEDICATION

I dedicate this work to my wife, Emily Burkhart, who has supported me throughout this endeavor.

And to my mother, Sandra Croom, and to Dr. Randy Rose. They gave me a good start but they could not make it to the finish.

BIOGRAPHY

Edward Lee Croom was born somewhere in North Carolina on March 10, 1975. Ed was adopted a month later and raised by his parents Sandra and Clyde Croom, who thirteen years later also adopted his sister Candice. Ed grew up in Raleigh and spent much of his time at the North Carolina Museum of Natural Sciences. Ed volunteered at the museum for six years as a Junior Curator, where he provided animal care and gave presentations. During this time Ed travelled to Panama to help Dr. Donna Wolcott collect and process the large land crabs native to that country.

Ed graduated from William G. Enloe high school after taking every available science course and was accepted to North Carolina State University. As his external learning experience, Ed along with one other person ran the Acrocanthosaurus exhibit at the N.C. State Fair. An estimated 100,000 people saw that award winning exhibit. Ed received a B.S. in Biology and a B.S. in History from North Carolina State University.

It was at N.C. State that Ed met his future wife, Emily Burkhart, who now laments how much she knows about chemicals in spite of her degree in Political Science.
Ed and Emily have four children; Abigail, Maizie, Charlotte and Willow. After graduation Ed worked as a middle-school science teacher for a couple of years before returning to the relative safety of a Toxicology lab. Ed joined the department of Environmental and Molecular Toxicology in 2004. Ed began his Ph.D. research under the direction of Dr. Randy Rose with the topic of polymorphisms and pesticide metabolism. Ed has served as the N.C. State student representative for the RTP Drug Metabolism Discussion Group.

In June of 2005, Ed's mother, Sandra Croom, died as a result of a recurrence of leiomyosarcoma. Dr. Rose was killed in a car crash in May of 2006. Ed continued his research under the direction of Dr. Hodgson and Dr. Wallace.

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I first want to thank my parents who surrounded me with books and knowledge and accepted a kid who would rather play with bugs than balls. I have to thank my wife Emily, who provided me with support, encouragement, the occasional deadline and periodic breaks from our darling children. It was all appreciated.

I would like to thank the faculty who provided me instruction and encouragement. I am grateful to the many post-docs, technicians and students who instructed me on techniques and provided time and sanity saving hints and suggestions. I appreciate the Lommel Lab for allowing me to use the Odyssey equipment. I was relieved to learn that at least one company, Xenotech, had someone working for them who 1) knew what the nuclear pellet was and 2) was willing to sell me something that would otherwise be thrown away. I would like to thank the office staff who kept me in mind and got things to the right place at the right time.

I am grateful to Dr. Rose for helping me start this research and to Dr. Wallace and Dr. Hodgson who allowed me to continue my research after Dr. Rose's tragic car crash. Dr. Hodgson, in particular must be thanked for dealing with yet another Ph.D. candidate. Dr. Hines has my appreciation for allowing me to participate in important research using precious samples and for setting a great example of how human samples should be studied. Lastly, I am deeply humbled by the actions of so many parents who made this research possible by making the painful choice to donate the organs of their children.

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GENERAL INTRODUCTION

Xenobiotics are foreign chemicals that are not normally produced by the organism in question or found in its normal metabolic pathways. Other organisms can produce xenobiotics and humans consume large numbers of xenobiotics in their diet. Xenobiotics are also produced from abiotic sources such as the radon released from uranium containing rocks. Humans are also exposed to an increasing number of xenobiotics which are chemically synthesized for consumer and industrial uses. Pharmaceuticals and pesticides are examples of some of the new xenobiotics created each year.

Xenobiotic exposures can be beneficial or detrimental. Pharmaceuticals are used to treat diseases and agrochemicals are used to facilitate the production of food and fiber. Often the dose determines whether an exposure to a xenobiotic is detrimental or beneficial. A high enough dose of any xenobiotic will inevitably cause toxicity.

The dose of a xenobiotic in the body can range from parts per trillion to parts per thousand. Intentional exposures to xenobiotics often result in higher doses than unintentional exposures. Intentional exposures often involve pharmaceuticals, food products, nutritional supplements, drugs of abuse and cosmetics. In some instances intentional exposures involve environmental chemicals. The term “environmental chemicals” is used to describe agricultural chemicals, environmental contaminants, industrial and household chemicals as well as chemicals found in natural ecosystems (Hodgson and Rose, 2007).

Most of our knowledge about human variability in xenobiotic metabolism is derived from the study of pharmaceuticals, which are often consumed in large doses. Unexpected

toxicities resulting from affected patients' inability to metabolize certain drugs were the impetus for the discovery that some variability in xeniobiotic metabolism is hereditary.

Toxicity is the capacity of a chemical to cause harm or death in a living organism. Toxicity involves more than just the interaction of the chemical with one target. Toxicity is the result of the entrance of the toxicant into the organism and multiple interactions between the toxicant and the organism as shown in figure 1.

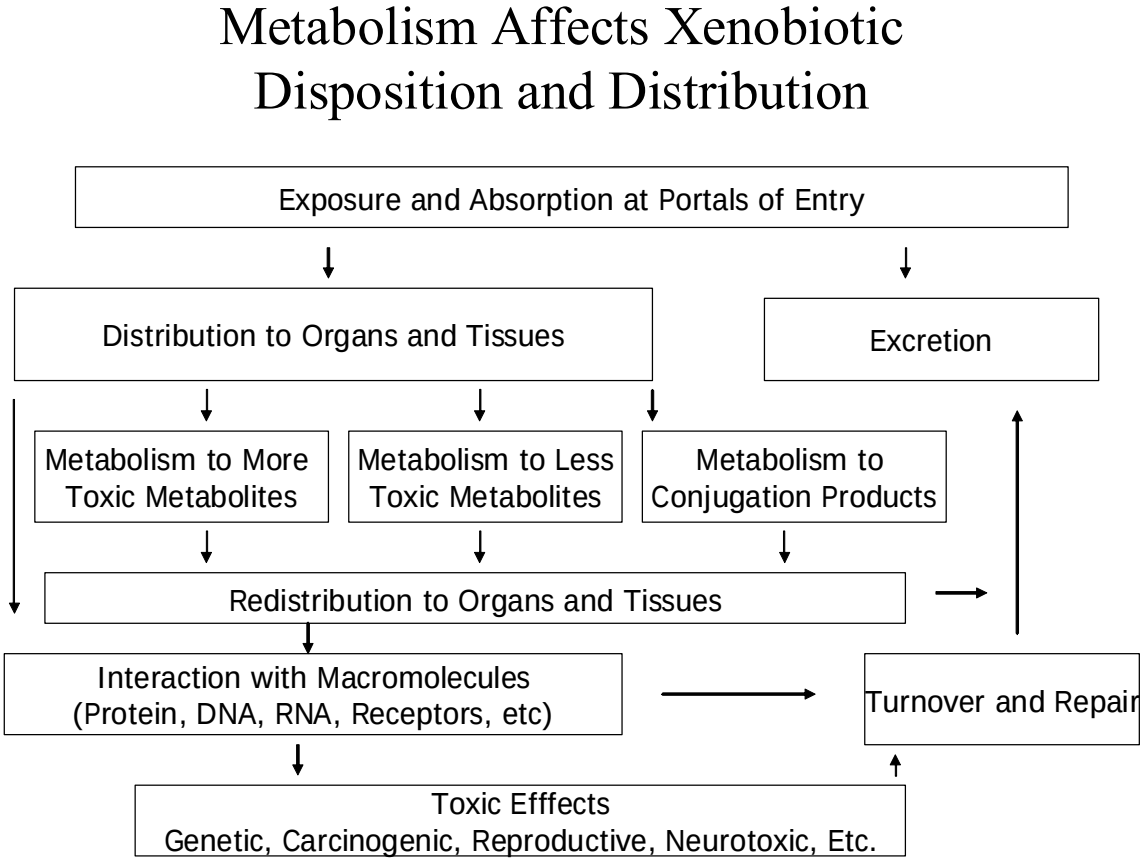

Figure 1 **Metabolism and Toxicant Exposure.**

The Globally Harmonized Standards classify chemicals into five acute toxicity groups based on the dose in mg/kg required to kill 50% of the animals tested; I.<5 mg/kg, II.<5-≤50 mg/kg, III.< 50-≤300 mg/kg, IV.<300-≤2000mg/kg and V.<2000-≤5000mg/kg (E.C.E., 2007). Category I compounds, such as Botulinum toxin, are extremely toxic and a few milligrams of these compounds can be fatal. Category III compounds, are moderately toxic compounds, but can cause fatalities even if less than 20 grams are ingested. Category V compounds, such as ethanol, have limited toxicity and must be consumed in huge quantities to cause death. Toxicity is often organism specific and can depend on the presence of different targets and the capacity to metabolize the toxicant.

Metabolism of xenobiotics can involve several reactions including oxidation, reduction, hydrolysis, hydrogenation and conjugation. Lipophilic chemicals are most likely to be absorbed and remain inside an organism for extended periods of time. Many xenobiotic metabolizing enzymes are designed to modify lipophilic chemicals into more hydrophilic compounds that are more readily excreted.

Phase I metabolism occurs when a polar reactive group, such as an oxygen atom, is added to a lipophilic xenobiotic. This can be a detoxification reaction or a bioactivation reaction. Phase II metabolism occurs when a xenobiotic or metabolite with a reactive group is conjugated with a hydrophilic endogenous metabolite. Transporters located in cell membranes are designed to facilitate the excretion of the conjugated metabolites.

The cytochromes P450 (CYPs) are major Phase I metabolizing enzymes. The CYPs have been detected in every eukaryote examined and may be essential for members of that domain. There are currently 7232 known CYPs, which are organized in 781 families (Nelson,

2008). Of the 110 animal CYP families, there are only 18 CYP families in vertebrates (Nelson, 2008). While most vertebrate CYPs metabolize endogenous substrates, four families in particular metabolize a large number of xenobiotics .

CYPs are hemoproteins named after their absorbance of light at 450 nm after the heme is reduced and bound by carbon monoxide (Omura and Sato, 1964). The heme group is essential to their catalytic activity and CYPs become inactive and absorb light at 420nm when the orientation of the heme is lost (Omura and Sato, 1964). CYPs function by oxidizing substrates using the reaction mechanism shown in figure 2.

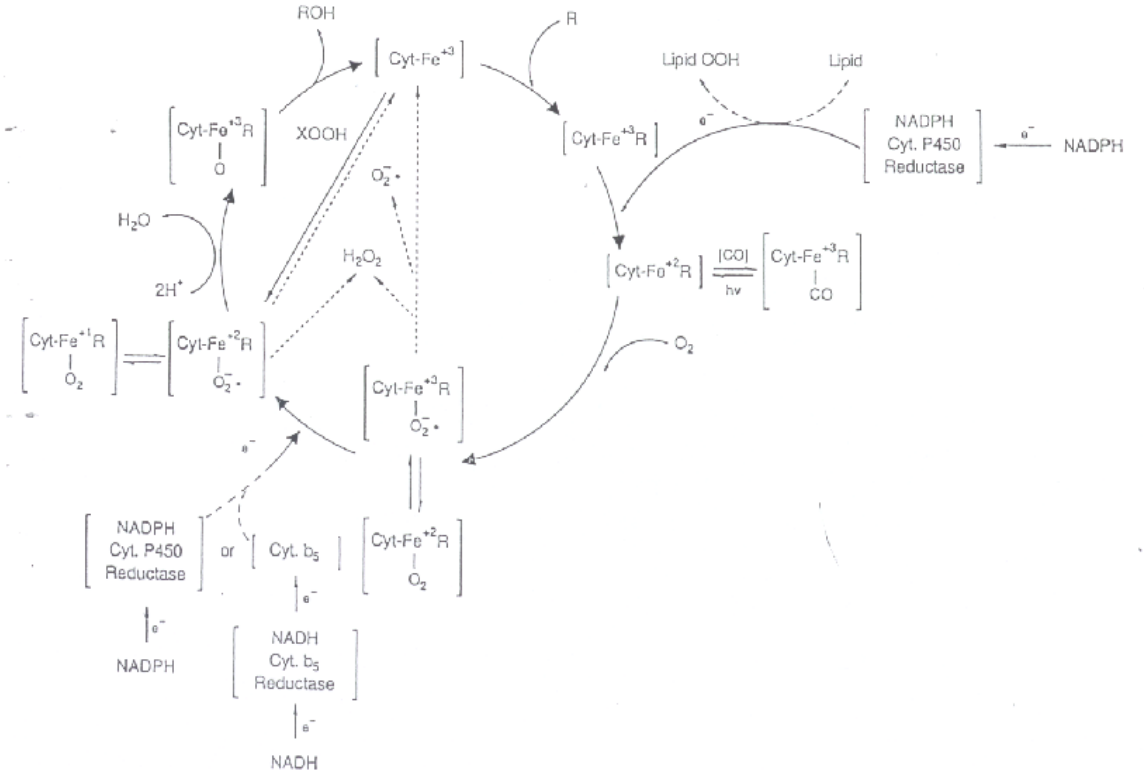

Figure 2 **CYP reaction cycle.**

CYP activity can be beneficial or detrimental depending on the substrate and its concentration. Oxygenation produces a nucleophile that can be conjugated or can react with

DNA and proteins to cause cellular damage. Phase II enzymes can conjugate these reactive intermediates but Phase II conjugation requires endogenous co-factors that may be consumed with high exposures. Liver necrosis caused by acetaminophen overdose is an example of a phase I metabolism overwhelming the capacity of conjugating enzymes to detoxify a reactive metabolite.

Acetaminophen is a moderately toxic analgesic. Acetaminophen, while safe when used as directed, can be fatal when used incorrectly and acetaminophen misuse is a leading cause of acute liver failure in the United States (James et al., 2008). Acetaminophen is primarily detoxified by glucuronidation. A small percentage of the drug is bio-activated by CYP2E1 into a toxic metabolite, *N* -acetyl-p-benzoquinone-imine (NAPQI). Normally NAPQI is detoxified through conjugation with a sulfate molecule from the sulfate donor 3'-phosphoadenosine 5'-phosphosulfate or by conjugation with glutathione. Unconjugated NAPQI can react with nearby liver tissue causing necrosis (James et al., 2008). Excess acetaminophen can reduce the glutathione concentration or that of the cofactors used in glucuronidation and sulfation, causing higher levels of NAPQI which can result liver damage and, in extreme cases, liver failure and death (James et al., 2008).

Chlorpyrifos (CPS) is a widely used organophosphorothioate insecticide (OP) (US EPA, 1989). CPS is sold under the trade names Brodan, Dursban, Eradex, Lorsban, and Spannit at concentrations up to 50% active ingredient. Indoor use of CPS was restricted in the United States in 2002 partly as a means to reduce childhood CPS exposures due to concerns about this population's susceptibility to toxicity (US, 1989). Between 2002 and 2006 approximately 3 million kilograms of CPS were applied each year in the United States,

primarily for agricultural uses (Williams et al., 2006). OPs such as CPS account for a large percentage of the total number of pesticide related poisonings worldwide and in some countries OPs are commonly used as a means of attempting suicide (Lin et al., 2008).

CPS acts as an insecticide by inhibiting AChE activity (AChE, EC 3.1.1.7), resulting in the accumulation of the neurotransmitter acetylcholine in neural junctions causing excessive firing of the nerves, resulting in cholinergic toxicity. CPS is a weak AChE inhibitor that derives most of its in vivo toxicity through bioactivation into its more toxic metabolite, chlorpyrifos-oxon (CPO)(Chambers, 1992). CPO is produced through a desulfuration reaction (Fig. 3).

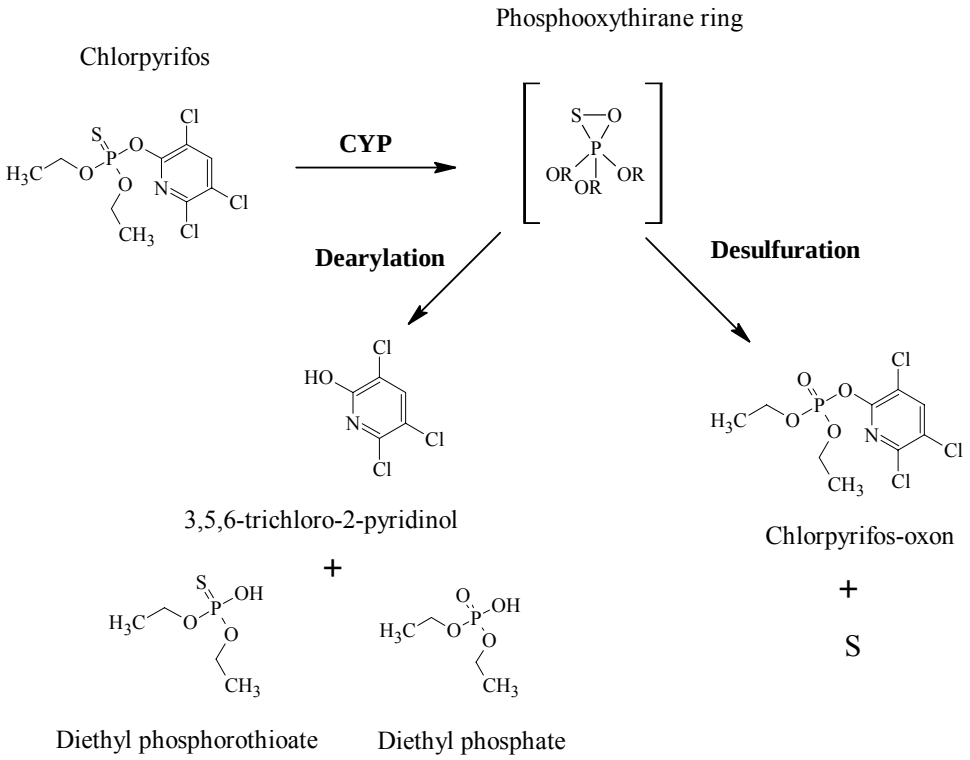

Figure 3 **Chlorpyrifos metabolism pathway.**

The CYPs are exclusively responsible for the production of CPO. CYP2B6 and CYP3A4 have been identified as major pesticide metabolizing isoforms (Hodgson and Rose, 2007). CYP3A4 and CYP2B6 are two major CPO producing isoforms in human liver microsomes (Tang et al., 2001). CYP3A4 and CYP2B6 are polymorphic, inducible and can vary greatly in expression and activity between and among individuals (Faucette et al., 2004). CYP3A4 has developmental delays in expression (Stevens et al., 2003). CYP3A4 has rare alleles that have been determined to alter CPS metabolism (Dai et al., 2001), but no common clinically relevant CYP3A4 variants are known. Limited information exists about the developmental expression of CYP2B6 protein, but in a study of 10 infant and fetal samples only two expressed detectable levels of CYP2B6 protein (Tateishi et al., 1997).

There is no information on the possible impact of CYP2B6 polymorphisms on pesticide metabolism. However, a common clinically relevant CYP2B6 polymorphism, the *CYP2B6*6* allele, has a frequency ranging from 15% to over 50% (Hofmann et al., 2008). The two mutations in the CYP2B6*6 allele have been found to lower CYP2B6 protein levels and alter the kinetics of the expressed CYP2B6.6 protein by increasing the activity towards some substrates and reducing the activity towards other substrates (Zanger et al., 2007).

CPS poisoning in humans occurs when the amount of CPO produced exceeds the capacity to detoxify CPO before it can inhibit AChE activity. CPO is detoxified by multiple esterases. Alpha esterases reversibly bind CPO which is then hydrolyzed and the enzyme restored. PON1 is a human alpha-esterase found in the blood. Beta-esterases irreversibly bind CPO and are found in many tissues including the blood, liver and central nervous

system (Furlong et al., 2006). Carboxylesterases, butyrylcholinesterases and AChEs are beta-esterases found in humans.

PON1 is the human esterase with the greatest CPO metabolizing activity (Furlong et al., 2006) Greater susceptibility towards CPS poisoning has been demonstrated in transgenic mouse models expressing the less active human PON1 (Q192) variant than mice expressing the more active human PON1 (R192) enzyme (Cole et al., 2005). PON1 is developmentally regulated. PON1 levels in children do not plateau until at least six months of age and often not until two years of age (Furlong et al., 2006). This delay in PON1 expression could result in greater susceptibility toward CPS poisoning in children. Furlong et al., (2006) reported an average 4-fold lower PON1 levels in neonate blood than PON1 levels in maternal samples and predicted a 164-fold range in CPO metabolizing capacity when combining the impact of age and poor metabolizer PON1 (Q192) genotype.

Young rats have been found to have greater susceptibility towards CPS poisoning than mature rats and this greater susceptibility has been determined to be the result of reduced esterase activity rather than increased bioactivation (Padilla et al., 2000). It is possible that humans with low PON1 levels due to age would also have sufficient ability to produce CPO and be poisoned by CPS exposures.

HYPOTHESIS

Age and genotype have been found to interact to create a subset of individuals with a lower than average capacity to detoxify CPO (Furlong et al., 2005). It is the hypothesis of this project that age and genotype will interact to reduce the capacity to produce CPO. To test

this hypothesis, the impact of the CYP2B6*6 allele on CPS metabolism and the hepatic developmental expression of CYP2B6 will be examined using human liver microsomes.

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CHAPTER 1

Human Variation in CYP-Specific Chlorpyrifos Metabolism.

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ABSTRACT

Chlorpyrifos, an organophophorothioate insecticide, is bioactivated to the neurotoxic metabolite, chlorpyrifos-oxon (CPO) by cytochromes P450 (CYPs). To determine the variability in chlorpyrifos bioactivation, CPO production by human liver microsomes from seventeen individual donors was compared to phenotype and genotype. CPO production varied over 14-fold between individuals in incubations utilizing 20μM chlorpyrifos as substrate, while CPO production varied 57–fold in incubations with 100μM chlorpyrifos. For all but two samples, the formation of the less toxic metabolite, 3,5,6-trichloro-2-pyridinol (TCP), was greater than CPO production. TCP production varied 9-fold in incubations utilizing 20μM chlorpyrifos as substrate and 19-fold using 100μM chlorpyrifos. Chlorpyrifos metabolism by individual human liver microsomes was significantly correlated with CYP2B6, CYP2C19 and CYP3A4 related activity. CPO formation was best correlated with CYP2B6 related activity at low (20μM) chlorpyrifos concentrations while CYP3A4 related activity was best correlated with CPO formation at high concentrations (100μM) of chlorpyrifos. TCP production was best correlated with CYP3A4 activity at substrate concentrations of both 20μM and 100μM chlorpyrifos. The production of CPO and TCP were both significantly lower at a concentration of 20μM chlorpyrifos as compared to 100μM chlorpyrifos. Calculations of percent total normalized rates (%TNR) and the chemical inhibitors ketoconazole and ticlopidine were used to confirm the importance of CYP2B6, CYP2C19, and CYP3A4 for the metabolism of chlorpyrifos. The combination of ketoconazole and ticlopidine inhibited the majority of TCP and CPO formation. CPO formation did not differ by CYP2B6 genotype. CPO production may need to be considered in determining the risk of chlorpyrifos poisoning.

*Keywords: c*hlorpyrifos; CYP isoform; desulfuration; dearylation; human liver microsomes; organophosphorothioate pesticides.

1. Introduction

Chlorpyrifos [*O*,*O*-diethyl-*O*-(3,5,6-trichloro-2-pyridinyl)-phosphorothioate] is a widely used organophosphorothioate insecticide (OP). Chlorpyrifos (CPS) acts by inhibiting acetylcholinesterase activity (AChE, EC 3.1.1.7), resulting in the accumulation of the neurotransmitter acetylcholine in neural junctions causing excessive firing of the nerves, resulting in cholinergic toxicity. Signs of cholinergic toxicity can include miosis, loss of bodily functions (salivation, lacrimization, urination and defecation), bronchospasm and, in extreme cases, death. Household use of CPS was banned in the U.S. in 2002, (EPA, 2001) and exposure levels in urban dwellings have declined since the ban (Williams et al., 2006). CPS continues to be widely used in agriculture, and occupational and dietary exposure to CPS have been documented (Hoppin et al., 2006; Lu et al., 2008). Lower level exposures to CPS have been associated with wheezing (Hoppin et al., 2006). In vitro studies have demonstrated the ability of CPS to inhibit neural development at levels lower than those associated with AChE inhibition (Howard et al., 2005; Sachana et al., 2008). CPS is also a potent CYP3A4 inhibitor and has been found to significantly inhibit estradiol and testosterone metabolism by human liver microsomes (HLM) (Usmani et al., 2003; Usmani et al., 2006). Epidemiological studies and in vivo research have also led to concerns about possible developmental delays resulting from CPS exposure (Levin et al., 2002; Whyatt et al., 2005; Eaton et al., 2008).

CPS inhibits AChE activity at relatively high concentrations (>5μM), but most of its in vivo toxicity occurs following oxidative desulfuration catalyzed by cytochromes P450s (CYPs). (Chambers, 1992; Buratti et al., 2003). The CYPs are proposed to activate CPS by forming an unstable phosphooxythiran intermediate that can be desulfurated or dearylated (Fig. 1). Desulfuration forms the potent AChE inhibitor, chlorpyrifos-oxon (CPO) while the detoxication product, 3-5-6-trichloro-2-pyridinol (TCP), is formed by dearylation (Chambers, 1992). Although CPS desulfuration is too rapid to allow the detection of this phosphooxythiran intermediate, every CYP found capable of CPO production also produces TCP, a finding which supports this proposed pathway (Tang et al., 2001; Sams et al., 2004; Buratti et al., 2006).

Multiple CYPs have been identified as CPS metabolizing enzymes. Results differ as to the capacity of CYP1A2, CYP2A6, CYP2C9 and CYP2D6 to metabolize CPS. In contrast, CYP2B6, CYP2C19 and CYP3A4 have consistently been identified as major CPS metabolizing isoforms (Tang et al., 2001; Buratti et al., 2003; Sams et al., 2004; Foxenberg et al., 2007). CYP2B6, CYP2C19 and CYP3A4 differ significantly in the relative amounts of CPO and TCP produced. CYP2B6 predominately forms CPO and CYP2C19 primarily forms TCP while CYP3A4 produces similar amounts of TCP and CPO (Tang et al., 2001). While CYP3A4 is generally more abundant than CYP2B6 and CYP2C19 in the human liver, it also has a lower affinity towards CPS than either CYP2B6 or CYP2C19 (Foxenberg et al., 2007).

These CPS metabolizing CYPs are polymorphic, inducible and their levels can vary greatly among individuals. Differing levels of these CYP isoforms may modify the risk of CPS intoxication. Variation in isoform specific activities has been associated with significant differences in CPS metabolism (Tang et al., 2001). Several mutations in the CYP genes have been found to impact drug metabolism raising the possibility that polymorphisms that affect drug metabolism may also impact CPS metabolism. *CYP2B6*6* and *CYP2C19*2* are common, clinically relevant, functional polymorphisms which have not previously been tested for their impact on CPS metabolism (Itoh et al., 1999; Hofmann et al., 2008).

While CYPs are exclusively responsible for CPO production, CPO detoxifying esterases are also capable of producing TCP through the dearylation of CPO. Human variation in paraoxonase 1 (PON1) levels has been well studied (Cole et al., 2005). Work with human PON1 and mouse models of OP intoxication has revealed that a common polymorphism found in the *PON1* gene impacts the ability of PON1 to protect against CPS intoxication (Cole et al., 2005). It is possible that CYP polymorphisms may also affect CPS metabolism by altering the amount of CPO produced.

In this report chemical inhibitors and phenotyped HLM were used to confirm the identity of isoforms shown to be responsible for CPS metabolism in vitro. The aims of the experiments described in this report were; 1) to compare CYP isoform specific activities to CPO and TCP production by single donor HLM, 2) to use chemical inhibitors to examine the relative importance of CYP2B6, CYP2C19 and CYP3A4 toward CPS metabolism and, 3) to

use genotyped HLM to examine the impact of the CYP2B6*6 allele on CPS metabolism in vitro.

2. Materials and Methods

2.1 Chemicals

CPS, CPO, and TCP were purchased from ChemService (West Chester, PA). High-performance liquid chromatography (HPLC) grade acetonitrile and methanol were purchased from Thermo Fisher Scientific (Waltham, MA). Ketoconazole and ticlopidine were purchased from Sigma-Aldrich (St.Louis, MO). SNP Genotyping assays; C_25986767_70 (g.19154G>A, rs4244285), C_7817765_60 (g.15631G>T, rs3745274), a previously designed custom assay, A785G (g.18053A>G, rs2279343) (Tsuchiya et al., 2004) and Taqman Genotyping Master Mix were supplied by Applied Biosystems (Foster City, CA). All other reagents, if not specified, were purchased from commercial sources at the highest grade available.

2.2 Microsomes

Supersomes, individual human recombinant CYP isoforms expressed in baculovirus-infected insect cells, containing CYP3A5, CYP3A4, CYP3A7, CYP2B6, CYP2C19, CYP2C9*1, CYP2C9*2, CYP2C9*3, CYP2C8, or vector only control microsomes were purchased from BD Biosciences (San Jose, CA). Single donor human liver nuclear pellets and single donor HLM were purchased from Xenotech (Lenexa, KA).

2.3 Metabolite detection

CPS and its metabolites, CPO and TCP, were separated by reversed phase HPLC on a C18 (4.6mm x 2.5cm i.d.) column from Phenomenex (Torrance, CA) and a C18 guard column (Phenomenex) using a method modified from Tang et al. (2001). The Shimadzu (Columbia, MD) HPLC system used for metabolite identification consisted of two pumps (LC-10ATVP), an autoinjector (SIL-10ADVP) and a controller (SCL-10AVP). Compounds were detected at 230nm using a UV-Vis detector (SPD-10AVP) and analyzed with VP 7.4 software (Shimadzu). An isocratic method with 67% acetonitrile, 32.5% water and 0.5% phosphoric acid was used with a flow rate of 1ml/minute.

2.4 Experimental conditions

Standards were diluted in reaction buffer and acetonitrile then stored at -20C between uses. Standards ranging from 0.025 to 10 nanomoles were used to generate standard curves. Limits of detection were determined following Environmental Protection Agency guidelines (CFR, 2006). The limit of detection for both CPO and TCP was 0.4µM. Retention times for CPO, TCP and CPS were 5.4, 7.7 and 14.0 minutes respectively. The inter-batch coefficient of variation for 0.4µM CPO was 6.4 (7 batches over two months).

The incubation mixture consisted of reaction buffer (50mM potassium phosphate buffer, pH 7.4, with 3.3mM $MgCl_2$ and 1mM EDTA), an NADPH regenerating system (0.25mM $NADP^+$, 2.5mM glucose-6-phosphate, and 2U/ml glucose 6-phosphate dehydrogenase) and CPS at a final concentration of 100µM or 20µM. Reactions were performed in 1.5 ml microcentrifuge tubes in a final volume of 250µl and were initiated by the addition of ice cold microsomes. Final protein concentration was 1.2 mg microsomal protein/ml. After ten minutes the reactions were terminated by adding 250µl of ice-cold acetonitrile and pulse-vortexing. The tubes were centrifuged at 20,000g for 5 minutes and the supernatant was transferred to clean vials. The supernatant was examined by HPLC-UV using the system described above. The injection volume was 15µl for reactions using 100µM CPS and 50µl for reactions using 20µM CPS.

2.5 Recombinant CYP Isoform incubations

Supersomes, individual recombinant CYP isoforms (rCYPs) expressed in baculovirus-infected insect cells, were incubated according to manufacturer's recommendations (BD Biosciences). All reactions contained the NADPH regenerating system and CPS at a final concentration of 100µM. A reaction buffer with 3mM $MgCl_2$ and 1mM EDTA was used for all supersomes. CYP2C9*1, CYP2C9*2, CYP2C9*3, were incubated in 100mM Tris-HCl buffer, pH 7.4. CYP3A4, CYP3A5, CYP3A7 were incubated in 50mM potassium phosphate buffer. CYP2B6, CYP2C8 and CYP2C19 were incubated in 100mM potassium phosphate buffer.

2.6 Correlation studies

Correlation coefficients (r) were calculated by comparing CYP isoform specific marker activities to rates of TCP or CPO production. The CYP isoform specific activities for the HLM used in this study, expressed as pmol/mg*min, are (range of values and mean ± SD); CYP1A2 phenacetin O-dealkylation 35-1930 (780 ± 467), CYP2A6 coumarin 7-hydroxylation 4-2930 (1329 ± 906), CYP2B6 bupropion hydroxylation 14-3840 (842 ± 1199), CYP2C8 amodiaquine N-dealkylation 297-6920 (2786 ± 2179), CYP2C9 diclofenac 4'-hydroxylation 365-4350 (2265 ± 1226), CYP2C19 S-Mephenytoin 4'-hydroxylation 6-319 (64 ± 79), CYP2D6 dextromethorphan O-demethylation 55-689 (265 ± 171), CYP2E1 chlorzoxazone 6-hydroxylation 698-6100 (2347 ± 1720), CYP3A4/5 testosterone 6ß-hydroxylation 43-19500 (4147 ± 5152), CYP3A4 midazolam 1'-hydroxylation 7-3990 (805 ± 1098), and CYP4A11 lauric acid 12-hydroxylation 438-3260 (1707 ± 743)(Xenotech, 2008).

2.7 Percent total normalized rate calculations

Total normalized rates (TNR) and % TNR values were calculated using the method of Rodrigues (1999) using the results of microsomal metabolism experiments provided by Xenotech (Xenotech, 2008) and kinetic constants calculated for recombinant isoforms (Foxenberg et al., 2007). The CPO or TCP formation rate (pmol/min*pmol recombinant CYP) was multiplied by the CYP isoform level (pmol/mg microsomal protein) in HLM samples, producing a normalized rate (NR) (pmol/min/mg microsomal protein). The NRs for each isoform involved in the production of the metabolite are summed to form the TNR. The %TNR is calculated using the following equation. % TNR = NR/TNR x 100

2.8 Chemical inhibition studies

Ketoconazole, a selective CYP3A4/5 inhibitor (Baldwin et al., 1995) was used at a concentration of 1µM. Ticlopidine, a mechanism based inhibitor, was used at a concentration of 10µM, a level shown to inhibit both CYP2B6 and CYP2C19 activity (Richter et al., 2004). Reactions contained 300µg protein/ml in a 250µl final volume. Ketoconazole and ticlopidine were dried down and dissolved in reaction buffer. Reactions

used the incubation mixture described above with 50mM potassium phosphate buffer. Inhibitors were pre-incubated for three minutes after the addition of ice-cold microsomes. CPS at a final concentration of 20µM was added to start the reaction. Reactions were terminated after 30 minutes by the addition of 250µl of ice-cold acetonitrile and pulse-vortexing. The tubes were centrifuged at 20,000g for 5 minutes and the supernatant was transferred to clean vials. The supernatant (50µl) was then examined by HPLC-UV using the system described above.

2.9 DNA extraction and genotyping

The first pellet formed during microsome preparation contains nuclei and other cellular debris. This nuclear pellet was purchased as a custom product from Xenotech. Aliquots of the nuclear pellet were allowed to self-digest for 30 minutes at 37C in Tris-HCl buffer then DNA was extracted using a phenol-chloroform method. SNP Genotyping Master Mix and Taqman SNP genotyping assays were used to determine the presence of the *CYP2B6*6* (G516T and A785G) and *CYP2C19*2* alleles according to manufacturers instructions. Assays were set-up in a PCR dedicated hood and run on an Eppendorf Mastercycler (Westbury, NY). A post-reaction read was performed using a 7300 RT PCR system (Applied Biosystems). DNA free negative controls were used for each run. Positive controls were DNA samples from donors previously identified by genotype and chosen to represent variant, referent and heterozygous genotypes respectively.

2.10 Statistical analysis

Groups were compared by the Mann-Whitney Rank Sum test using Systat's SigmaPlot program (San Jose, CA). Pearson's correlation coefficient was calculated for linear regression analysis (SigmaPlot). Linear regression was used to assess the range of metabolite levels, develop the standard curves and quantify unknown metabolite levels. In all cases $p < 0.05$ was considered significant. For correlation analysis, a cut-off value of $r > 0.60$ was used (Rodrigues, 1999).

3. Results

3.1 Variation in CPS metabolism among single donor HLM

Every single donor HLM sample produced both TCP and CPO from 100µM CPS (Fig 2A). Every single donor HLM sample formed TCP from 20µM CPS and all but one sample formed detectable levels of CPO from 20µM CPS (Fig 2B). The mean ratio of CPO/TCP formation was 0.5±0.4 from 20µM CPS and 0.7±0.3 from 100µM CPS. The variation between donors in CPO and TCP formation found in this study was greatest at 100µM CPS but remained high at 20µM CPS. Activity at 100µM CPS correlated with activity at 20µM ($r2 = 0.56$, $p<0.001$). TCP formation from 100µM CPS varied 19-fold from 91 to 1,706 pmol/mg*min while CPO production varied 57-fold from 12 to 681 pmol/mg*min among single donor HLM. TCP formation from 20µM CPS varied 9-fold from 43 to 420 pmol/mg*min. Among the 16 HLM samples producing detectable amounts of CPO from 20µM CPS, CPO production varied 14-fold from 17 to 237 pmol/mg*min.

3.2 CPS metabolism by recombinant human CYP isoforms

CYP2C19, CYP3A4, CYP2B6 were most active towards CPS metabolism. CYP2C19 produced the most TCP, while CYP2B6 formed the most CPO. CYP3A4 formed slightly more TCP than CPO (Table 1). CYP2C9.1, CYP3A5, and CYP3A7 had some activity towards CPS while CYP2C9.2, CYP2C9.3 and CYP2C8 did not metabolize CPS.

Using CYP isoform specific activity information provided by Xenotech, TCP formation was found to correlate best with CYP3A4 activity at both 20µM and 100µM CPS (Table 2). CPO formation correlated best with CYP2B6 activity at 20µM CPS and CYP3A4 activity at 100µM CPS (Fig.3). CYP2B6, CYP2C19 and CYP2C8 activities also correlated significantly with TCP formation. CPO formation correlated with CYP2C8 activity. The inactive CYP2C8 isoform also correlated with CYP2B6 activity ($r2 = 0.69$) and CYP3A4 activity ($r2 =0.64$) for these samples presumably as a result of co-expression of the isoforms.

3.3 Total normalized rates

The metabolism of CPS by single donor HLM and the contribution of CYPs based on specific activities were used to determine the %TNRs. Calculations of TNRs identified CYP2B6, CYP3A4 and CYP2C19 as the major CPS metabolizing isoforms for most donors

(Table 3). CYP2B6 and CYP3A4 were the major contributors to CPO production. CYP2C19 and CYP3A4 were the major contributors to TCP production. CYP2C9 and CYP1A2 played only a minor role in CPS metabolism.

3.4 Effect of chemical inhibition on CPS metabolism

The CYP chemical inhibitors ketoconazole and ticlopidine were found to inhibit TCP and CPO production by all seventeen single donor HLM samples incubated with 20μM CPS (Table 4). Inhibition of CPO formation by ticlopidine weakened the correlation between CYP2B6 specific activity and CPO formation. Ticlopidine use also increased the strength of the correlation between CYP3A4 specific activity and CPO formation. Neither ticlopidine nor ketoconazole alone completely inhibited TCP and CPO production. The combination of ticlopidine and ketoconazole inhibited a greater percentage of TCP and CPO production than either ticlopidine or ketoconazole used as single inhibitors. The combined use of ticlopidine and ketoconazole significantly increased the correlation of CPO production with CYP2A6 activity and CYP2C9 activity.

3.5 Genotype and CPS metabolism

Single donor HLMs were genotyped to determine the presence of the CYP2B6*6 and CYP2C19*2 alleles (Table 5). Single donor HLMs with the *CYP2B6*6/*6* genotype did not differ significantly from single donor HLMs with the *CYP2B6*1* genotype in the production of TCP or CPO from either 100μM CPS or 20μM CPS (Fig. 4). The single donor HLM sample with the *CYP2C19*2/*2* genotype produced the most CPO from 20μM CPS of any sample.

4. Discussion

Risk assessment for regulatory purposes relies almost exclusively on data from surrogate animals, however surrogate animals cannot be used predict human variation, an essential component of risk assessment. Studies of human variation are, consequently, an important adjunct to current risk assessment. Using single donor HLMs we determined that the metabolite TCP was formed by all seventeen samples from a 20μM CPS substrate concentration and all but one sample produced CPO from 20μM CPS. Greater amounts of TCP than CPO were produced for all but two samples, consistent with findings from other studies of the human metabolism of CPS (Tang et al., 2001; Sams et al., 2004; Buratti et al., 2006). Lowering the CPS concentration in HLM incubations was found to greatly reduce the rate of desulfuration and dearylation for all samples (Fig. 1). This supports the earlier indications that CPS is metabolized by high affinity (low Km) and low affinity (high Km) enzymes. The weak correlation between CPO production from substrate concentrations of 100μM and 20μM CPS (r2 = 0.56) also supports the role of higher affinity enzymes at lower substrate concentrations.

Using rCYPs and single donor HLMs, CYP3A4 and CYP2B6 were identified as the major CPO producing isoforms and CYP3A4 and CYP2C19 were identified as the major TCP producing isoforms in this study. Tang et al. (2001), Sams et al. (2004), Mutch and Williams (2006), and Foxenberg et al. (2007) also identified CYP2B6 as a major CPO producing isoform. Tang et al. (2001), Mutch and Williams (2006), and Foxenberg et al. (2007) also identified CYP3A4 and CYP2C19 as the most active TCP producing isoforms.

CYP2C9, CYP3A5, CYP3A7 and CYP1A2 also metabolized CPS. Other studies differ on the CPS metabolizing capacity of these isoforms and others, including CYP2E1, CYP2D6, and CYP2A6. Different results for isoform activity from previous studies might be partly due to the use of different methods and different substrate concentrations. Buratti et al. (2003) used a method that does not permit TCP detection. Substrate levels in CPS metabolism studies have ranged from a low of 3μM CPS (Foxenberg et al.,2004) to a high of 100μM CPS.

Little is known about the possible effects of CYP polymorphisms on CPS metabolism in humans. Some recently identified mutations in the CYP genes have been studied for their impact on the CPS metabolism. Tang et al. (2001) studied CYP2C19.5, CYP2C19.6 and CYP2C19.8. They all had significantly reduced capacity to produce TCP and none produced detectable levels of CPO. CYP3A4*17 had lower CPS turnover than wild-type CYP3A4, while CYP3A4*18 had higher activity than wild-type CYP3A4 (Dai et al., 2001; Tang et al., 2001). CYP3A4*17 (Lee et al., 2005) and CYP3A4*18 (Lee et al., 2007) have been found among the Adygei ethnic group and individuals of Asian ancestry respectively but have not been detected among other populations (Dai et al., 2001).

CYP3A5 and CYP3A7 metabolized CPS confirming the results of an earlier study (Buratti et al., 2006). CYP3A7, while primarily a fetal and neonatal isoform (Stevens et al., 2003), is polymorphic in expression among adults. The low activity of CYP3A7 towards CPS may limit its significance in adults, but the high expression of CYP3A7 in the fetal liver could make it significant in the fetus. CYP3A5 was slightly less active towards CPS than CYP3A4, but would only be relevant in individuals expressing this gene.

CYP2C9.2 did not metabolize CPS confirming earlier results (Tang et al., 2001). The present study also found no activity towards CPS from CYP2C9.3. One of the two samples with low TCP compared to CPO production was the sample with the CYP2C19*2/*2 genotype. Since CYP2C19 produces more TCP than CPO, this could be due to the loss of CYP2C19 specific activity towards CPS, but the lack of additional samples with the CYP2C19*2/*2 genotype renders these findings inconclusive.

CYP3A4 represented the most abundant CYP isoform for most of the donors. However, CPO production correlated best with CYP2B6 activity in experiments using a concentration of 20μM CPS, but to CYP3A4 activity at 100μM CPS. This results from the higher affinity of CYP2B6 towards CPS. Foxenberg et al. (2007) reported a Km for CPS desulfuration by CYP2B6 of 0.81μM and a Km for CYP3A4 of 27μM.

CYP3A4 activity was found to correlate well with TCP production at both concentrations, but CYP2C19 correlated better at 20μM CPS. The Km for CPS dearylation by CYP3A4 is 33.4μM, while the Km for CPS dearylation by CYP2C19 is 1.6μM

(Foxenberg et al., 2007). The Vmax for CYP2C19 did not change at a lower concentrations of CPS, but CYP3A4 showed a reduction in velocity at lower concentrations in earlier studies (Sams et al., 2004). Mutch and Williams (2006) did not identify CYP2B6 as a major CPS metabolizing isoform in their correlation studies; however the CYP2B6 specific activity did not correlate to the CYP3A4 specific activity as expected.

While current methods have not allowed the detection of TCP production from lower levels of CPS using HLM, more sensitive methods using AChE inhibition as a marker of CPO production have been used. CYP2B6 activity was best correlated to CPO formation from 5μM CPS (Buratti et al., 2003) supporting the current study's finding that CYP2B6 activity became more important to predicting CPO production at lower CPS concentrations.

CYP2C8 activity correlated well with TCP and CPO formation by HLM from 100μM CPS, although lymphoblast expressed CYP2C8 does not metabolize CPS (Tang et al., 2001). Since CYP2C8 is induced by similar mechanisms as CYP2B6 and CYP3A4, co-regulation explains this association between CYP2C8 specific activity and CPS metabolism (Ferguson et al., 2005). The lack of correlation between CYP2C9 specific activity and CPS metabolism found in this study confirms previous results (Buratti et al., 2003) that found no link between CPO production and CYP2C9 activity.

Ketoconazole alone inhibited less than half of TCP and CPO production. Ticlopidine inhibited less than half of TCP production and around 50% of the CPO production from HLM. Sams et al. (2004) also reported that no single CYP isoform selective inhibitor inhibited the majority of CPS metabolism by HLM (Sams et al., 2004). Combining both ticlopidine and ketoconazole inhibited the majority of CPS metabolism but was not completely additive. The use of either ketoconazole or ticlopidine significantly increased the correlation of CYP2A6 activity with CPO production. Sams et al. (2004) identified CYP2A6 as a CPS metabolizing isoform. The combined use of ketoconazole and ticlopidine significantly increased the correlation of CYP2C9 activity with CPO production. These results suggest that minor CPS metabolizing isoforms such as CYP2A6 and CYP2C9 may become more important when CYP2B6 and CYP3A4 activity is low as a result of inhibition.

Ketoconazole did not increase the correlation between CYP2B6 activity and CPO production. In contrast, ticlopidine significantly increased the correlation between CYP3A4 activity and CPO production, implying that CYP3A4, with a lower affinity, remains unsaturated. Both ketoconazole and ticlopidine significantly inhibited CPS metabolism by most HLM samples, but the amount of inhibition varied greatly (Table 4). A similar degree in variability was found in the % TNR for individual isoforms (Table 2).

CYP2B6 genotype did not predict CPO production in this study. The 516G>T mutation present in the *CYP2B6*6* allele, is associated with the formation of a splice variant and subsequent reduction in protein expression (Hofmann et al., 2008). It is possible that induction may be able to increase CYP2B6 expression in *CYP2B6*6/*6* individuals up to at least 80pmol/mg microsomal protein (Hofmann et al., 2008). The five CYP2B6*6/*6 samples did not differ significantly in buproprion hydroxylation activity, a CYP2B6 specific activity, when compared to CYP2B6*1/*1 samples. This lack of effect may be explained by the small sample size and inability to control for exposures to CYP2B6 inducers. Given the high variability among samples a sample size of 66 was predicted as being required for adequate power to compare the two genotypes.

Only CYPs are responsible for bioactivating CPS. In vitro studies have consistently identified CYP2B6 as a major CPO producing isoform (Tang et al., 2001; Buratti et al., 2003; Sams et al., 2004; Foxenberg et al., 2007). CYP2B6 could be the most significant CPO producing isoform at low, toxicologically relevant, concentrations of CPS for several reasons. CYP2B6 has the highest affinity and highest intrinsic clearance towards CPS desulfuration (Foxenberg et al., 2007). CYP2B6 protein levels can vary over 350-fold and its hepatic levels can reach ~ 180 pmol/mg microsomal protein (Hofmann et al., 2008). Lastly, CYP2B6 protein has been identified in the human brain, the critical site in CPS poisoning (Miksys et al., 2003).

In conclusion, CPS metabolism varied significantly between individual samples and correlated with CYP3A4, CYP2C19 and CYP2B6 activity. Work with rCYP isoforms, %TNR calculations, and chemical inhibition studies support the identification of CYP2B6, CYP2C19 and CYP3A4 as the major CPS metabolizing isoforms. At lower CPS

concentrations CYP2B6 activity best predicted CPO production. Individual differences in CYP2B6 expression may result in greater variability in risk from CPS exposures than is accounted for solely based on esterase levels. Individuals with high CYP2B6 levels but low esterase levels may be at greatest risk.

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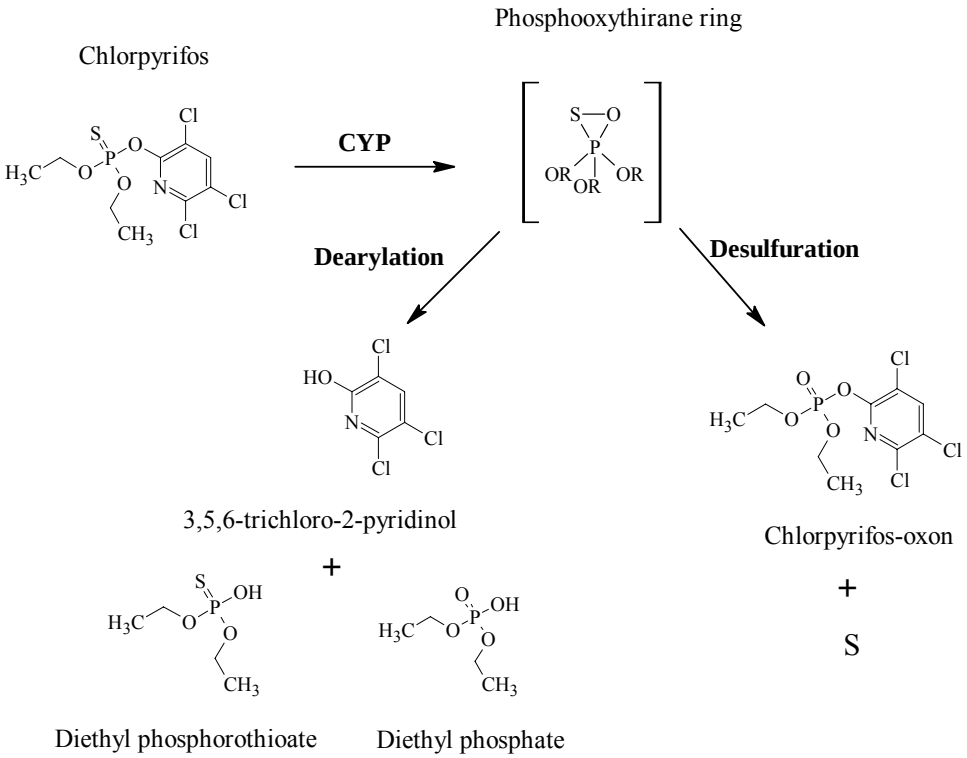

Figure 1 **Proposed pathway for microsomal metabolism of chlorpyrifos.** The formation of an unstable intermediate could result in either desulfuration or dearylation.

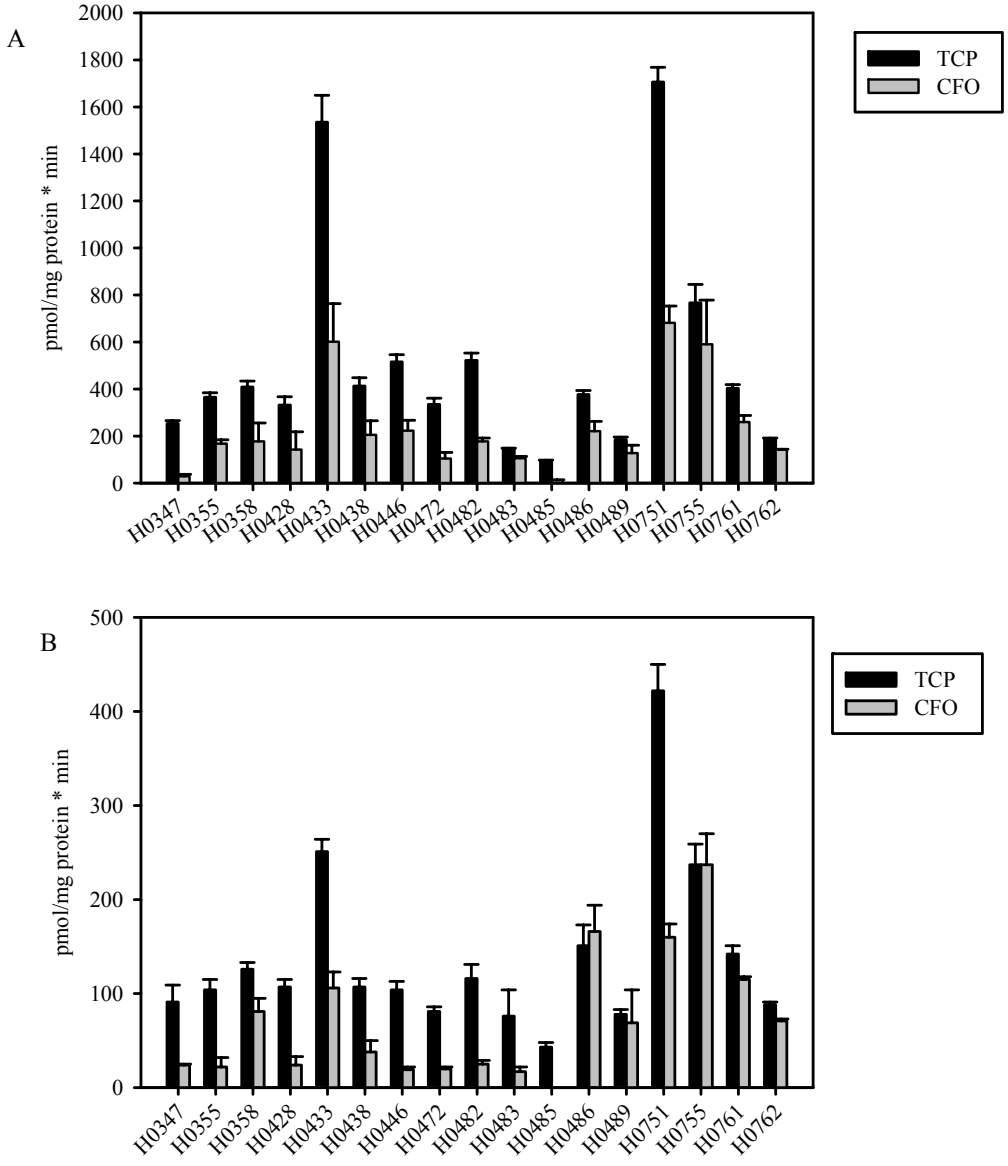

Figure 2. **Formation of TCP and CPO by single donor human liver microsome samples.** Seventeen single donor samples were treated with 100µM chlorpyrifos (A) and 20µM chlorpyrifos (B).

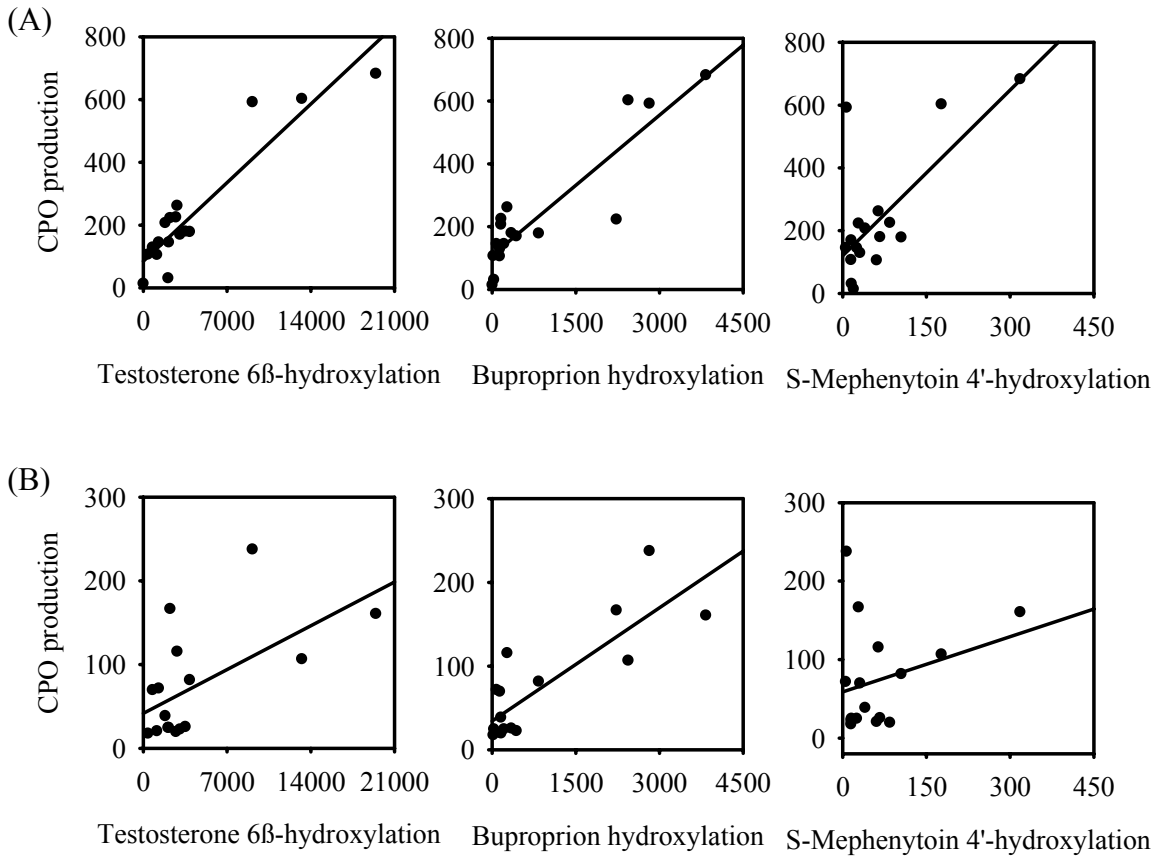

Figure 3 **CYP isoform specific activities correlated to chlorpyrifos-oxon formation.** CYP isoform specific activities correlated to chlorpyrifos-oxon formation from **(A)** 100µM chlorpyrifos and **(B)** 20µM chlorpyrifos.

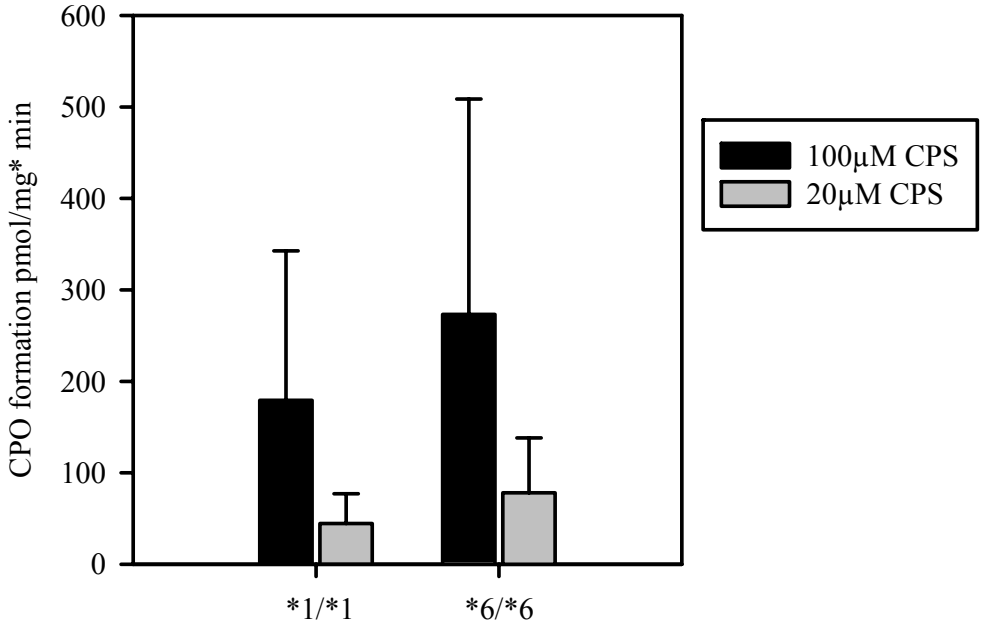

Figure 4 **CYP2B6 genotype and the formation of chlorpyrifos-oxon.**

Table 1. **Ratio of chlorpyrifos desulfuration to dearylation by human CYP isoforms.**

CYP isoform	Desulfuration/Dearylation
CYP2B6	2.54
CYP2C9*1	1.21
CYP2C19	0.21
CYP3A4	0.77

Table 2. **Correlation between CYP isoform specific activities and chlorpyrifos metabolism in human liver microsomes.**

CYP marker activity[a]	Coefficient of determination (r^2)			
	TCP (20μM CPS)	TCP (100μM CPS)	CPO (20μM CPS)	CPO (100μM CPS)
Phenacetin O-dealkylation (CYP1A2) [b]	0.11	0.12	0.03	0.18
Coumarin 7-hydroxylation (CYP2A6)	0.24	0.21	0.27	0.35 *
Bupropion hydroxylation (CYP2B6)	0.86 ***	0.71 ***	0.69 ***	0.80 ***
Amodiaquine N-dealkylation (CYP2C8) [b]	0.71 ***	0.65 ***	0.48 **	0.79 ***
Diclofenac 4'-hydroxylation (CYP2C9)	0.18	0.1	0.35 *	0.24
S-Mephenytoin 4'-hydroxylation (CYP2C19)	0.70 ***	0.77 ***	0.08	0.49 ***
Dextromethorphan O-demethylation (CYP2D6)	0.02	0.06	$2x10^{-8}$	0.03
Chlorzoxazone 6-hydroxylation (CYP2E1)	0.31	0.15	0.34	0.27
Testosterone 6ß-hydroxylation (CYP3A4/5)	0.95 ***	0.95 ***	0.35 *	0.85 ***
Lauric acid 12-hydroxylation (CYP4A9/11)	0.26	0.15	0.06	0.15

[a] All CYP isoform marker activities (range of values and means ± S.D.) are reported under Materials and Methods. Range of values for TCP and CPO formation are reported in Results.
[b] N = 14. For all other activities N = 17.
* $p<0.05$, ** $p<0.01$, *** $p<0.001$

Table 3. **Percentage total normalized rates (%TNR) of chlorpyrifos metabolism calculated for seventeen individual HLM.**

	%TNR	
CYP isoform	TCP production	CPO production
CYP1A2	4±3	5±3
CYP2B6	6±6	35±17
CYP2C9	8±6	3±4
CYP2C19	19±15	4±6
CYP3A4	63±17	53±15

Table 4. **Effect of selective CYP inhibitors on chlorpyrifos biotransformation in single donor HLM.** Results are the average of duplicate experiments using seventeen individual donor HLM samples (mean ± SD, n = 17).

	Inhibition (%)	
Inhibitor (CYP inhibited)	TCP production	CPO production
Ticlopidine (2B6, 2C19)	34 ± 26	53 ± 30
Ketoconazole (CYP3A4)	36 ± 19	45 ± 29
Ticlopidine + Ketoconazole (2B6, 2C19, 3A4)	51 ± 18	67 ± 17

Table 5. **Genotypes of HLM donors.** Nuclear pellets from 49 HLM donors were tested for the presence of the CYP2C19*2 (rs4244285) and CYP2B6*6 (rs3745274, rs2279343) polymorphisms. HLM donors with homozygous variant genotypes were selected and paired with heterozygous and homozygous referent donors similar in age and sex.

ID	CYP2B6	CYP2C19
H0347	*1/*1	*1/*1
H0355	*1/*1	*1/*2
H0358	*1/*1	*1/*1
H0428	*1/*1	*1/*1
H0433	*1/*1	*1/*1
H0438	*1/*1	*1/*1
H0446	*1/*1	*1/*1
H0472	*6/*6	*1/*2
H0482	*6/*6	*1/*2
H0483	*1/*1	*1/*1
H0485	*1/*1	*1/*2
H0486	*1/*6	*1/*1
H0489	*1/*1	*1/*1
H0751	*6/*6	*1/*1
H0755	*1/*6	*2/*2
H0761	*6/*6	*1/*1
H0762	*6/*6	*1/*1

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CHAPTER 2

Human Hepatic CYP2B6 Developmental Expression: The Impact of Age and Genotype

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ABSTRACT

Although CYP2B6 is known to metabolize numerous pharmaceuticals and toxicants in adults, little is known regarding CYP2B6 ontogeny or its possible role in pediatric drug/toxicant metabolism. To address this knowledge gap, hepatic CYP2B6 protein levels were characterized in microsomal protein preparations isolated from a pediatric liver bank (N=217). Donor ages ranged from 10 weeks gestation to 17 years of age with a median age of 1.9 months. CYP2B6 levels were measured by semi-quantitative western blotting. Overall, CYP2B6 expression was detected in 75% of samples. However, the percentage of samples with detectable CYP2B6 protein increased with age from 64% in fetal samples to 95% in samples from donors >10 years of age. There was a significant, but only 2-fold increase in median CYP2B6 expression after the neonatal period (birth to 30days postnatal) although protein levels varied over 25-fold in both age groups. The median CYP2B6 level in samples over 30 postnatal days to 17 years of age (1.3 pmol/mg microsomal protein) was lower than previously reported adult levels (2.2 to 22 pmol/mg microsomal protein), however, this likely relates to the median age of these samples, *i.e.*, 10.3 months. CYP2B6 expression did not vary significantly by gender. Furthermore, CYP2B6 levels did not correlate with CYP3A4, CYP3A5.1 or CYP3A7 activity, consistent with different mechanisms controlling the ontogeny and constitutive expression of these enzymes and the lack of significant induction in the pediatric samples.

1. Introduction

CYP2B6 is the only functional member of the human *CYP2B* family and was originally thought to be absent in most individuals. Improved antibody preparations have now demonstrated the presence of immunoreactive CYP2B6 protein in the livers of most adults tested [1], although interindividual differences are among the largest of the cytochromes P450 that have been studied. Thus, a recent meta-analysis reported that CYP2B6 and CYP3A5 had the lowest minimum expression levels (1.0 pmol/mg microsomal protein). However, CYP2B6 exhibited maximum expression levels (45 pmol/mg microsomal protein) comparable to CYP1A2, 2A6, 2C8, and 2E1 (52 to 68 pmol/mg microsomal protein) and higher than either CYP2C19 (20 pmol/mg microsomal protein) or CYP2D6 (11 pmol/mg microsomal protein) [2]. This conclusion also is consistent with relative abundance levels. The relative abundance of CYP2B6 (0.4 to 8.4%, 21-fold range) and 3A5 (0.4 to 22%, 55-fold range) exhibited a much larger range than any of the other cytochromes P450 (all less than 3-fold range). Similar to the CYP2C and CYP3A family members, a portion of the interindividual variability in CYP2B6 expression may be explained by the ability of both the constitutive androstane (CAR, NR1I3) and pregnane X (PXR, NR1I2) receptors to induce CYP2B6 expression several fold in a ligand-dependent manner [3]. Although CYP2B6 is considered primarily a hepatic enzyme, it has been detected at lower levels in several other organs, including the brain [4], kidney and lung [5].

CYP2B6 participates in the oxidative metabolism of numerous pharmaceuticals, including the anti-depressant bupropion [6], the anesthetics propofol [7] and lidocaine [8],

the chemotherapeutic agents cyclophosphamide, ifosfamide [9] and tamoxifen [10], the anti-retroviral agent efavirenz [11] and the anti-malarial drug artemesinin [12]. CYP2B6 also plays a role in the metabolism of methadone [13] and the drugs of abuse, nicotine [14] and ecstasy [15]. Environmental contaminants such as styrene [16] and several pesticides, including chlorpyrifos [17] and endosulfan [18] are also excellent substrates. CYP2B6 also plays a role in the metabolism of endogenous substrates including the steroid testosterone [19]. Like other members of the cytochrome P450 family of proteins, CYP2B6 can detoxify and facilitate the elimination of toxicants and drugs, however, depending on the chemical properties of the substrate, CYP2B6-dependent oxidation also can increases the toxicity of several compounds, *e.g.*, tamoxifen, aflatoxin B1 [10] and chlorpyrifos [17]. Most important for the subject of this study, purposeful or accidental childhood exposures to all of these compounds have been documented.

CYP2B6 functional polymorphisms have been identified but null alleles are rare. The most clinically relevant polymorphism is the *CYP2B6*6* allele (g.15631G>T, rs3745274; g.18053A>G, rs2279343). The g.15631G>T transversion in the *CYP2B6*6* allele is predicted to result in the loss of a splice enhancer and is linked to the formation of transcript variant predicted to encode a non-functional protein [20]. However, because the *CYP2B6*6* allele exhibits incomplete penetrance, it is associated with reduced protein expression and reduced in vivo metabolism, rather than a complete loss of function [21].

Little is known about CYP2B6 expression during development. An early study of age-dependent CYP2B6 expression found lower levels in infant liver samples when

compared to adults [22], but this study included just two fetal samples and eight infant samples. Samples from individuals ranging from 2 to 72 years of age were not sufficient in number to permit a determination of any further temporal changes in CYP2B6 expression.

The objective of this study was to characterize CYP2B6 developmental expression and determine the possible impact of genetic variation on this process. The absence of an in vivo probe for CYP2B6 activity suitable for use in children, the difficulty in collecting pharmacokinetic data from infants, and the ethical issues preventing the study of in vivo fetal CYP2B6 metabolism necessitated the use of postmortem human liver microsomal samples. Comparing CYP2B6 expression with previously characterized CYP3A levels in the same samples also was performed in an attempt to gain some information regarding possible regulatory mechanisms.

2. Material and Methods

2.1 Materials

Polyclonal antibodies against CYP2B6 and lymphoblast-expressed CYP2B6 were purchased from BD Biosciences (San Jose, CA). IR800 dye-labeled goat anti-rabbit IgG antibodies were purchased from LI-COR (Lincoln, NE). Nitrocellulose membranes were supplied by Bio-Rad (Hercules, CA). EZ-Run prestained recombinant protein molecular weight markers, methanol and Tris-glycine buffer were obtained from Thermo Fisher Scientific (Waltham, MA). *CYP2B6* single nucleotide polymorphism (SNP) genotyping assays; C_25986767_70 (g.19154G>A, rs4244285), C_7817765_60 (g.15631G>T, rs3745274), C_26201809_30 (g.6986A>G, rs776746), a previously designed custom assay to detect A785G (g.18053A>G, rs2279343) [23] and Taqman Genotyping Master Mix were supplied by Applied Biosystems (Foster City, CA). All other reagents were purchased from Sigma-Aldrich (St. Louis, MO).

2.2 Human liver samples

Individual human liver microsomal samples (N=217) were obtained from donors ranging in age from 10 weeks gestation to 17 years of age. The median donor age was 1.9 months. There were 56 prenatal, 39 neonatal (birth to 30 days postnatal age) and 122 samples over 30 days postnatal age. Of the samples over 30 days postnatal age, 63 were less than one year of age. The source of pediatric liver samples and the preparation of fetal and postnatal microsomal samples were described previously [24]. DNA samples were prepared

as previously described [25]. Individual adult human liver microsomes (HK23) were purchased from BD Biosciences (San Jose, CA).

2.3 Electrophoresis and immunoblotting

SDS polyacrylamide gel electrophoresis was performed according to the method of Laemmli [26] using a Tris-glycine buffer, pH 8.3. Human liver microsomal proteins (10 to 40 μg per lane) were separated on 10% precast Novex gels and transferred to nitrocellulose membranes using a Transblot apparatus (Bio-Rad). Efficient transfer was verified with Ponceau S staining of membranes. Membranes were blocked with 5% non-fat dry milk in phosphate buffered saline (PBS)/Tween 20 (0.1% V/V) for 90 minutes at room temperature and incubated with primary antibodies overnight at 4ºC. Anti-CYP2B6 antibodies (BD Gentest) and anti-actin antibodies (Sigma) were used at 1:500 and 1:1000 dilutions, respectively. After 4 washes in PBS/Tween 20 (0.1% V/V), the membranes were incubated with the secondary antibody for 1 hr at room temperature in the dark. The secondary antibody, IR 800 labeled goat anti-rabbit IgG (LI-COR), was used at a 1:1250 dilution. Images were captured using the Odyssey Infrared Imaging System (LI-COR), software version 1.2. Lymphoblast-expressed CYP2B6 containing microsomes (BD Biosciences) were used to derive a standard curve using 31, 63, 125, 250 and 500 femtomoles of CYP2B6 protein. The pre-stained molecular weight markers served as a negative control. An adult human liver microsomal sample, HK23 (BD Biosciences), containing seven pmoles CYP2B6/mg microsomal protein was used as a positive control. The limit of detection was defined as three times the background intensity.

2.4 Genotyping

DNA samples were amplified using Taqman SNP genotyping assays and SNP Genotyping Master Mix (Applied Biosystems). Using 96 well optical plates and following manufacturer instructions, DNA and reagents were combined in a hood dedicated to polymerase chair reaction (PCR) set-up. Following an initial denaturation step of 95C for 10 mins, amplification was performed for 50 cycles of 92C for 15s and 60C for 90s. Reactions were run on a Mastercycler (Eppendorf ,Westbury, NY) and after amplification, results were read on a 7300 RT PCR system (Applied Biosystems). DNA free negative controls were used for each experiment. Positive controls were DNA samples from donors previously genotyped using PCR- restriction fragment length polymorphism and chosen to represent variant, referent and heterozygous genotypes respectively.

2.5 Statistical Analysis

Linear regression was used to assess the dynamic range of response for the standard curve, and subsequently, to quantitate CYP2B6 levels in microsomal samples. Coefficients of determination (r^2) of 0.90 or above were accepted. Statistical analysis of correlations between CYP2B6 and CYP3A protein levels were performed using JMP version 7.0 (SAS, Cary, NC). Data was log transformed before partitioning analysis was used to search for age groups differing in CYP2B6 protein levels. Logworth values > 1.3 and *p*-values <0.05 were considered significant. Groups were compared with the Mann-Whitney Rank Sum test or Kruskal-Wallis nonparametric ANOVA with a Dunn's post hoc test using SigmaPlot version

11.0 (Systat, San Jose, CA). Two-way ANOVA with a Holm-Sidak post hoc test were use to examine interactions. Box and Whisker plots were created using SigmaPlot.

3. Results

3.1 CYP2B6 Immunoquantitation

A single immunoreactive protein was detected by western blot in the pediatric microsomal samples that comigrated with lymphoblast expressed CYP2B6 and the adult positive control sample (Fig.1A). CYP2B6 protein levels were below the limits of detection (10 fmol/lane, 0.25 pmol/mg protein) in 25% of the microsomal samples, even though Ponceau S staining of the western blot showed even transfer and similar protein levels for all samples. To verify the presence of microsomal protein in the absence of CYP2B6 expression, a small number of blots were incubated with anti-actin antibodies and equivalent amounts of actin were confirmed for each sample (Fig.1B). Detectable CYP2B6 protein was observed in most samples from donors with ages throughout gestation and the postnatal period examined, although there was considerable interindividual variation (Fig.2).

To determine whether or not there was any significant change in CYP2B6 expression as a function of age, a partitioning analysis was performed and a break-point identified at the end of the neonatal period, i.e. 30 days after birth (log worth value 3.8). Samples from donors with a postnatal age greater than 30 days had median CYP2B6 levels (1.3 pmol/mg microsomal protein, range = 0.0 to 23.9 pmol/mg microsomal protein) approximately 2-fold higher than samples from younger donors (0.6 pmol/mg microsomal protein, range = 0.0 to 36.7 pmol/mg microsomal protein) [Mann- Whitney (p<0.001)] (Fig.3). The percentage of individuals with detectable levels of CYP2B6 protein also increased with age such that

CYP2B6 protein was detectable in 64% of the fetal samples (N=56) while after 6 months postnatal age (N=67), 90% of the samples expressed protein levels above the limit of detection. This trend continued with age, as 95% of the samples from donors 11 to 17 (N=20) years of age had detectable CYP2B6 protein levels (Fig.4). Both the percentage of samples with detectable CYP2B6 and the median CYP2B6 levels for these samples are significantly lower than reported adult median levels (Table 1). Only a small percentage of samples had CYP2B6 levels above the median adult levels (Fig.5).

3.2 Genotyping

Somewhat surprisingly, there was no difference in CYP2B6 protein levels between individuals genotyped as *CYP2B6*6/*6* (N=16) versus *CYP2B6*1/*1* (N=104) in either of the age groups (fetal to 30 days postnatal age versus older than 30 days) (Fisher's Exact Test).

3.3 Correlation of CYP2B6 and CYP3A levels

Because of some shared regulatory mechanisms reported in adults [27], CYP2B6 levels were compared to the levels of CYP3A7, CYP3A5 and CYP3A4 determined in a previous study using these same tissue samples [28]. No correlation was observed between CYP3A4, CYP3A5 or CYP3A7 and CYP2B6 expression levels (Fig. 6).

3.4 Correlation of CYP2B6 levels with ethnicity and sex

CYP2B6 expression was compared among African American (N=82), European American (N=93) and Hispanic American samples (N=18). Other ethnic groups were represented by too few samples for analysis. Samples from African American donors were

found to have lower levels of CYP2B6 than European American samples [Kruskal-Wallis, with Dunn's post hoc ($p<0.05$)]. However, this may be explained by the observation that African American donors had a significantly lower median age (1.7 months) than European American donors (3.4 months) [Mann-Whitney ($p<0.05$)]. CYP2B6 expression did not differ significantly between females (N=77) and males (N=132) [Mann-Whitney ($p<0.90$)]. Finally, CYP2B6 levels did not correlate with post-mortem intervals (time between death and freezing of tissue samples), consistent with previous reports on other enzymes using these same samples [24, 25, 28, 29].

4. Discussion

In an earlier report, CYP2B6 protein was detected in only 2 of 10 liver microsomal samples from donors > 37 weeks gestation but <10 months of age (mean ± SD = 2.7 ± 5.9 pmol/mg microsomal protein), but was found in 7 of 10 samples from donors ranging in age from 2 to 72 years (mean ± SD = 19.4 ± 23.9 pmol/mg microsomal protein) [22]. Although this same trend was observed in the current study, CYP2B6 levels above the limit of detection were observed in 64% of all fetal (N=56) and samples from birth to 30 days postnatal age (N=39) and increased to nearly 95% of samples with detectable CYP2B6 protein in samples from donors between 11 and 17 years of age (N=20) (Fig. 4). The more frequent detection of CYP2B6 in the current study probably is due to a much larger sample size and a lower limit of quantification based on the availability of improved antibody preparations.

Among those samples with detectable CYP2B6 levels, expression varied approximately 75-fold in the samples over 30 days postnatal age and 25-fold in the samples younger than 30 days postnatal age once a fetal outlier with 4-fold greater expression than any other sample in that age group was removed from the analysis. This fold variation is considerably less than that reported in an earlier meta-analysis [30]. Also, studies using adult samples have reported a maximum CYP2B6 level two to four times higher than the maximum CYP2B6 level observed in the current study of pediatric liver samples (Table 1). The lower levels of CYP2B6 observed in these pediatric samples is likely due to the purposeful weighting of the sample set to the period from birth to one year of age (median age of all postnatal samples = 3.5 months, median age of samples older than 30 days postnatal = 10.3 months), although fewer exposures to agents known

to induce CYP2B6 expression cannot be ruled-out as contributing to this observation. Consistent with the latter premise, several earlier studies included in the meta analysis described in Table 1 incorporated data from donor samples known to be exposed to CYP2B6 inducing agents, such as phenytoin and alcohol [1, 31]. Also consistent with the latter premise, the lack of correlation between CYP2B6 and CYP3A4 expression in the postnatal samples and the lower degree of interindividual variation would be consistent with the observed CYP2B6 levels representing constitutive rather than induced expression.

CYP2B6 levels increased with age with partitioning analysis revealing a significant approximate 2-fold difference between CYP2B6 levels in the samples from younger donors (10 weeks gestation to 30 postnatal days) versus those from older donors (>30 days to 17 years). A similar increase around one month after birth has been reported for other enzymes (*e.g.*, CYP2E1), although the magnitude of this increase appears greater for many of the other enzymes studied [25].

Induced CYP2B6 expression in adult humans is controlled in part by one or more nuclear receptors, including the PXR (NR1I2), CAR (NR1I3) and glucocorticoid receptor (NR3C1) [3, 27]. Regulation by these receptors and their activating ligands likely explains the observation that while some individuals have less than 1 pmol/mg microsomal protein of CYP2B6 present in their livers [19, 20, 31, 32], CYP2B6 levels can be over 350-fold higher in other individuals [20, 31]. These same nuclear receptors also can induce CYP3A4 expression, but the relative capacities of different ligands to induce CYP2B6 and CYP3A4 varies [27]. The expression of CAR or PXR proteins in human fetal liver is unknown, but it is possible as both CAR and PXR transcripts have

been detected in both fetal and pediatric liver samples [33]. A single study has addressed possible age-dependent changes in CAR expression wherein CAR transcripts and protein were detected in neonatal (birth to 30 days postnatal age) samples at lower levels relative to adults [34]. However, this study was underpowered in that only three neonatal samples were used to make this comparison. Work with human hepatocytes has shown the ability of phenobarbitol and rifampicin to induce CYP2B6 expression in individuals as early as 2 and 3 years of age (Rose RL, unpublished results)[27], suggesting functional PXR and/or CAR nuclear receptors by at least this age. However, for the reasons discussed above, it would appear induction by these receptors contributed little or none to the differences in CYP2B6 expression reported in the current study. Only 10% of the older samples (>30 days to 17 years) and 5% of younger samples (10 weeks gestation to 30 days postnatal) had CYP2B6 levels above 5 pmol/mg (Fig.5) providing further evidence that a change in constitutive CYP2B6 expression was involved in the age-related increase in median CYP2B6 levels.

CYP2B6 is highly polymorphic, but only rare alleles have been reported to eliminate expression. The *CYP2B6*6* allele has clear clinical relevance for substrates such as efavirenz and neverapine and has been shown to impact the metabolism of both compounds in children [35, 36]. The explanation for a failure to observe an impact of the *CYP2B6*6* allele in the current data set is puzzling, but may be explained by an age-dependent impact of the affected splicing enhancer on transcript processing.

CYP2B6 expression did not differ significantly by sex in the pediatric tissue samples. While sex differences in CYP2B expression have been well documented in

laboratory animals [37], such sexual dimorphic patterns in gene expression are more common and quantitatively greater in rodent species relative to the human (*e.g.*, CYP2C, FMO3). Sex differences in CYP2B6 levels have been reported. However, these differences may be confounded by exposures to CYP2B6 inducers and the small sample sizes utilized. For example, it has been reported that Hispanic females have higher CYP2B6 expression, but only three female and four male donors were used to infer that conclusion [38]. Other studies are consistent with the observations in the current study and have failed to find significant differences between males and females [20]. However, the paucity of samples older than 11 years of age (i.e., puberty) limits this conclusion.

Predicting effective doses for CYP2B6 therapeutic substrates can be difficult because of the highly inducible and polymorphic nature of the enzyme and potential complications resulting from both higher and lower CYP2B6 activity are a concern, including for the pediatric patient population [35]. For example, a pediatric patient receiving ifosfamide developed seizures after taking phenytoin and based upon a metabolic profile, drug-induced CYP2B6 induction was a suspected cause [39]. In another example, a reported childhood case of efavirenz-induced psychosis was suspected of being linked to the patient possessing a single copy of the *CYP2B6*6* allele [36]. Although a direct causal link to CYP2B6 was not made in either of these case-reports, these examples highlight the need to better understand and predict CYP2B6 metabolic ability. Such a need extends to a better understanding of CYP2B6 ontogeny, which is at least partially addressed in the current study. Thus, the ability to express CYP2B6 appears to exist throughout development for most individuals, although there appears to be considerable interindividual variability as to when the onset of

expression is observed. Further, there is a modest, but significant increased level of CYP2B6 expression after the neonatal period (>30 days postnatal age). The large individual variability in expression observed in adults was less apparent among pediatric samples, although this was likely due to the lack of CYP2B6 induction in most of the samples studied. Importantly, while these data clearly indicate the ability of older children to metabolize CYP2B6 substrates, consistent with in vivo case reports, (*e.g.*, [36]), these results also suggest that many infants, neonates and fetuses also may possess the ability to catalyze CYP2B6-dependent oxidation reactions.

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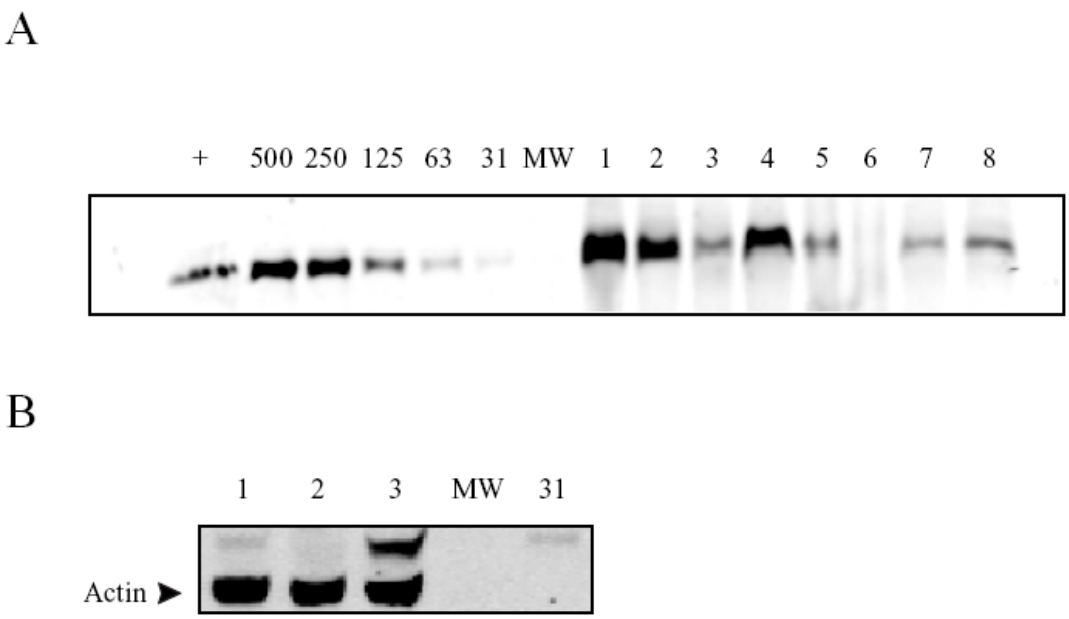

Figure 1 Detection of CYP2B6 by western blot. (A) A representative western blot showing the relative amounts of CYP2B6 protein from 40µg microsomal protein in a random set of individual pediatric-human liver microsome preparations. Expressed recombinant CYP2B6 (31 to 500 fmol/lane) was used to establish a standard curve. A previously characterized adult microsomal protein sample (+) and the molecular weight markers were used as positive and negative controls, respectively. While sample 6 had no detectable CYP2B6, samples 1, 2 and 4 exceeded the limits of the standard curve and were re-run with only 10µg protein. (B) A comparison of actin and CYP2B6 levels. The arrow indicates the actin protein. CYP2B6 levels ranged from 5 pmol/mg microsomal protein in sample 3 to below the limits of detection (0.25 pmol/mg microsomal protein) in sample 2.

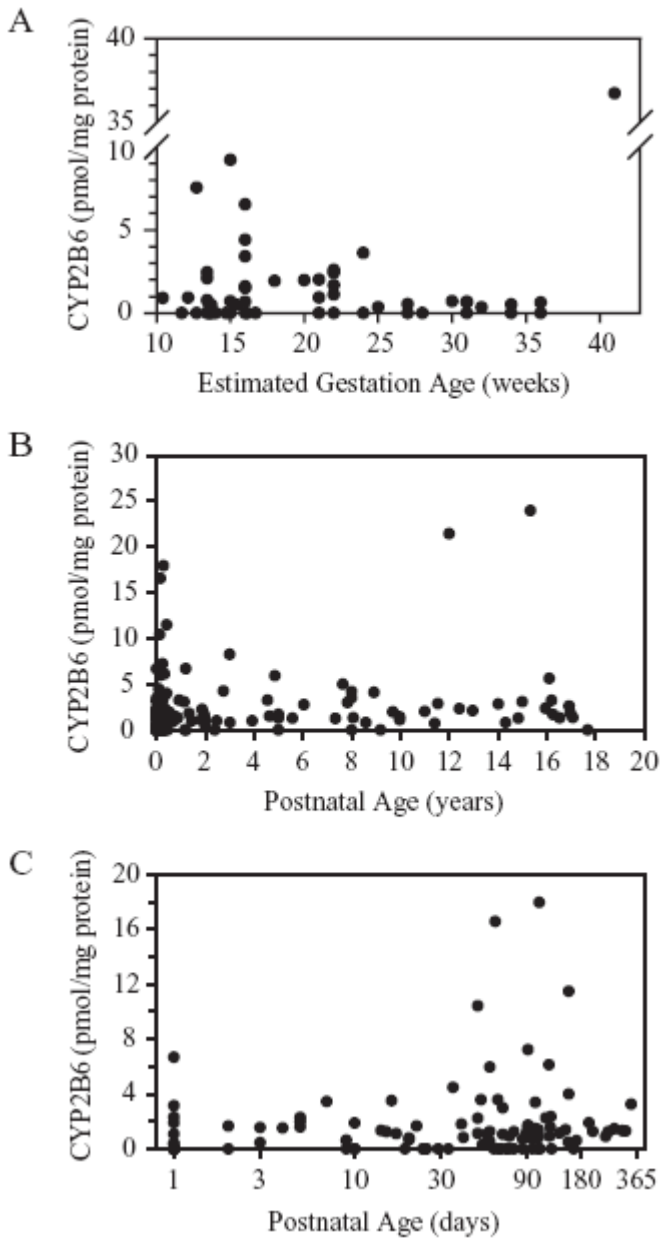

Figure 2 CYP2B6 expression in individual tissue samples. CYP2B6 expression in individual tissue samples is shown for (A) prenatal samples, (B) postnatal samples and (C) samples from birth to 1 year of age. Note that the postnatal age for graph (C) is on a log scale to better display the changes in CYP2B6 expression after 30 days of age.

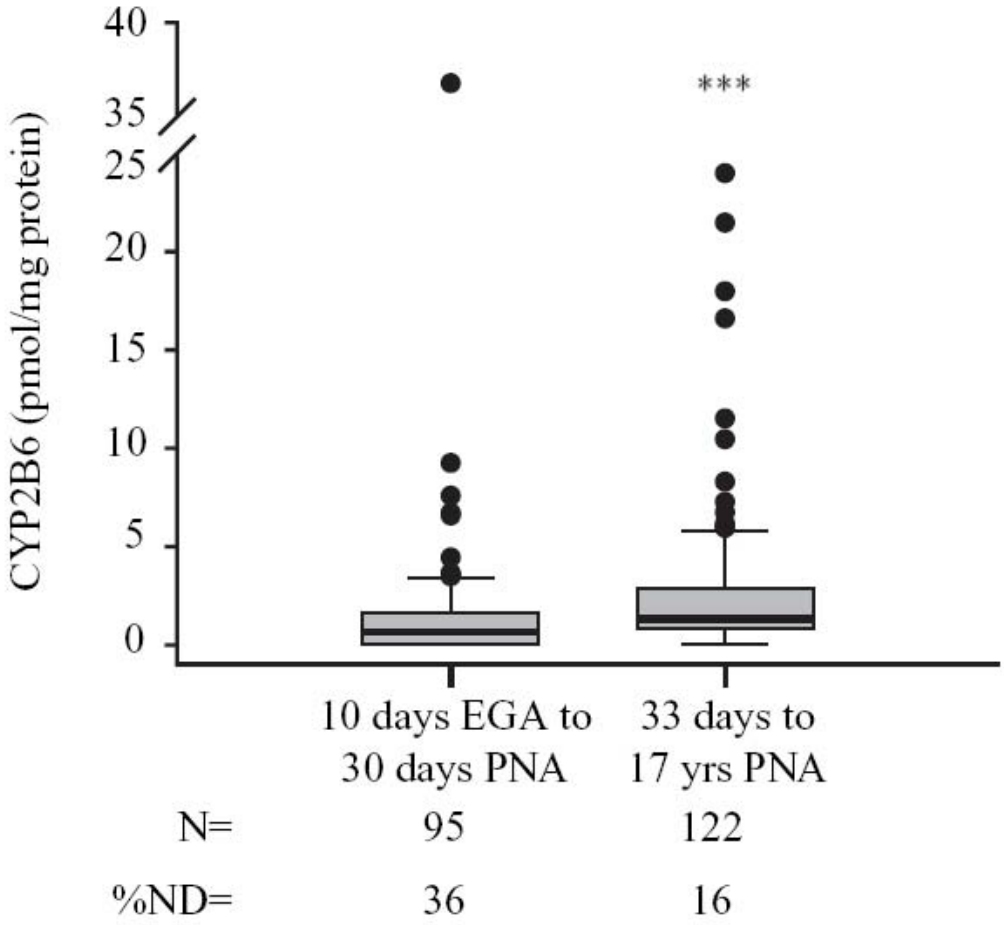

Figure 3 Increased CYP2B6 expression after neonatal period. Age brackets were chosen based on partitioning analysis using 217 samples. Boxes represent the interquartile values. The line represents the median value and the whiskers represent the 10th and 90th percentile values. Individual data points represent outliers (1.5-times the interquartile values). EGA = estimated gestational age, PNA = postnatal age.

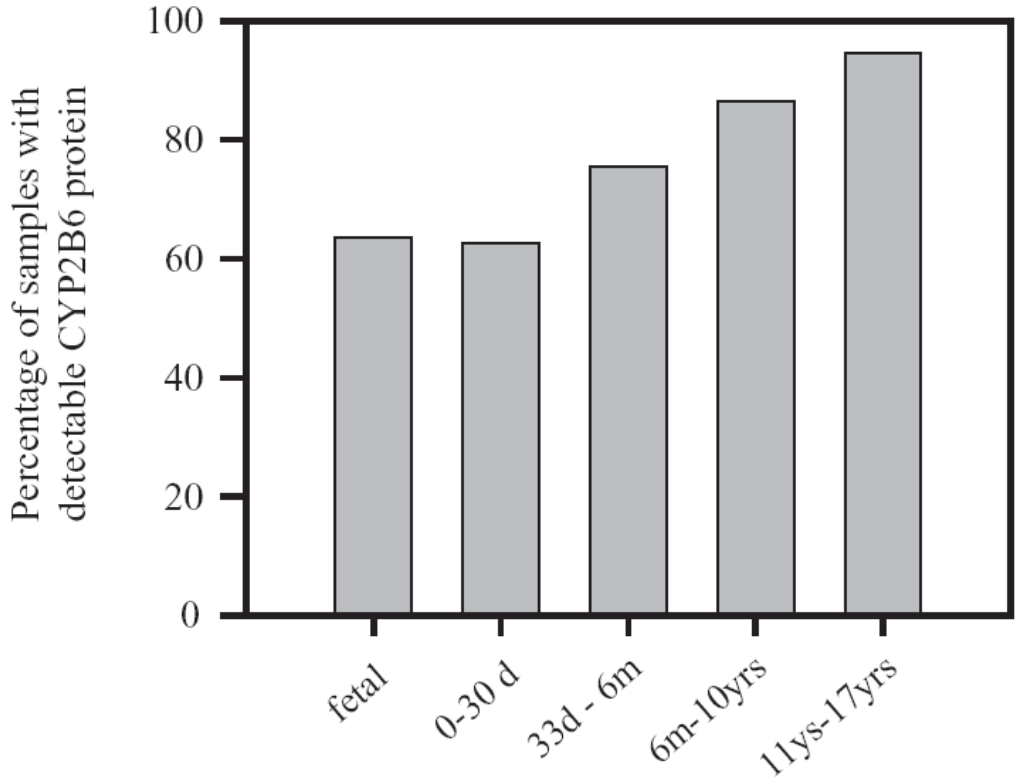

Figure 4 Percentage of samples with detectable CYP2B6 protein. The percentage of samples in the indicated age groups with detectable levels of CYP2B6 is shown. Fetal (N=56), 0-30 days postnatal (N=39), 31 days-6 months (N=55), 6 months-10 years (N=47), 11 years-17years (N=20).

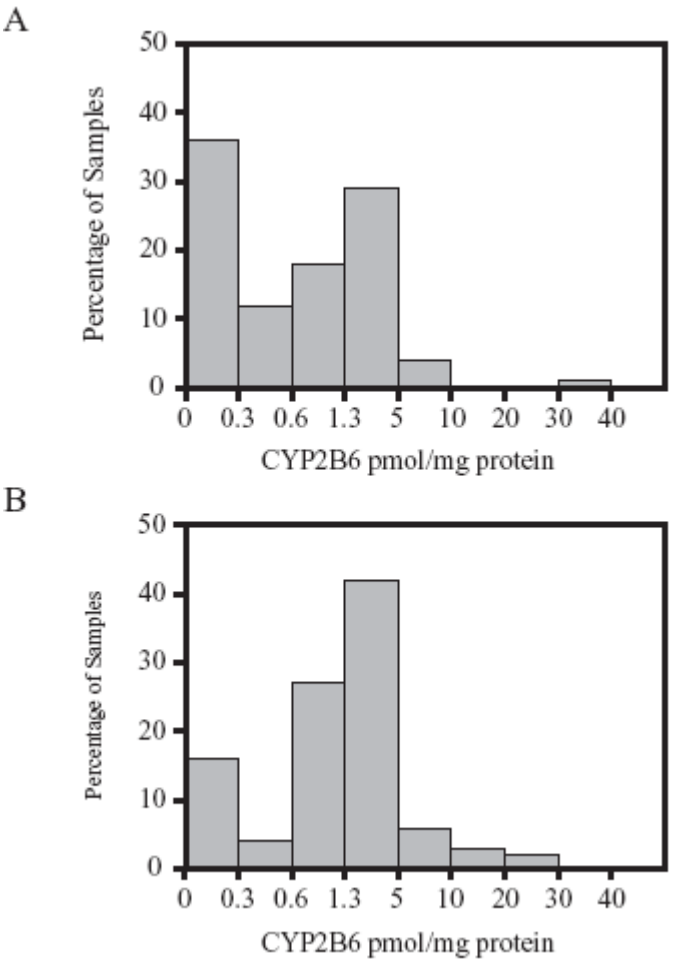

Figure 5 Distribution of CYP2B6 expression. The percent of samples with various levels of CYP2B6 expression levels in fetal/neonatal samples (10 weeks gestation to 30days postnatal age) (A) and samples older than 30 days postnatal age (B) is displayed in histograms. An expression level less than 0.3 pmol/mg microsomal protein was below the limit of detection. Samples with over 5 pmol CYP2B6/mg microsomal protein had levels above median adult CYP2B6 levels (Table 1).

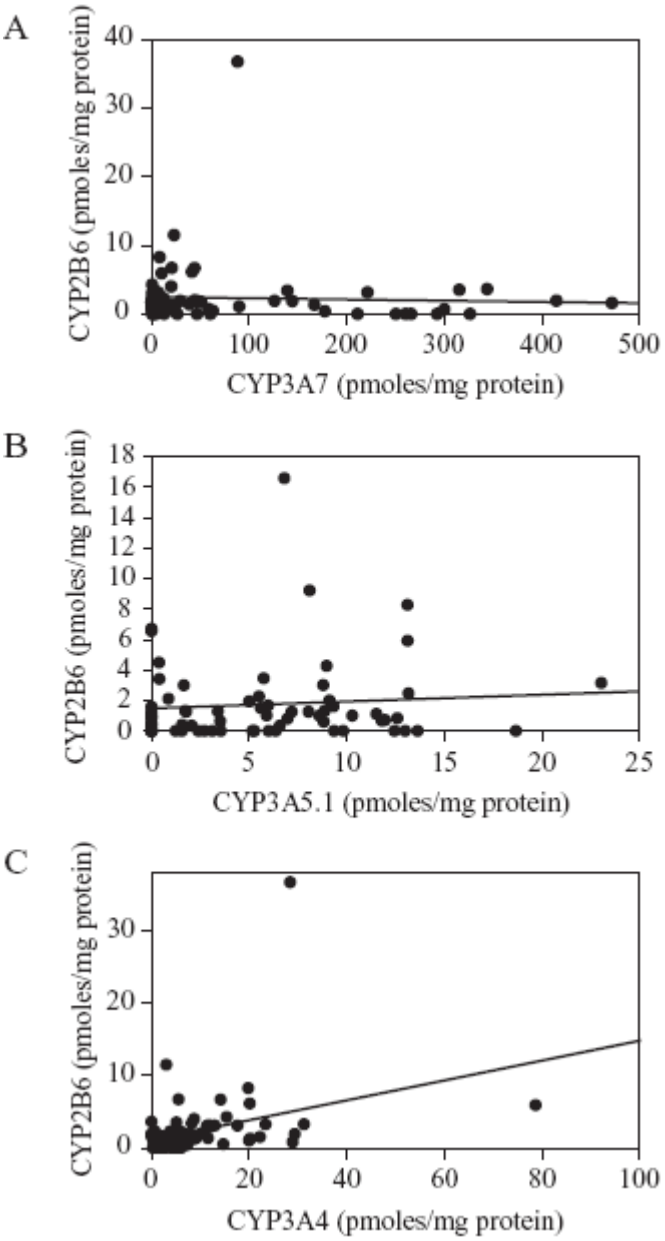

Figure 6 Correlation between CYP2B6 and CYP3A expression. The correlation between CYP2B6 and CYP3A5*1 (A), CYP3A7 (B) or CYP3A4 (C) expression in individual samples is depicted.

Table 1. **Comparisons of CYP2B6 Hepatic Expression.**

Detection (%)	pmol/mg protein median (range)	age in years median (range)	Variability	References
107/112 (96)	4 (0.3-82) a	41 (2.5-71)	273-fold	Meta analysis *b*
101/122 (83)	1.6 (0.3-23.9) a	0.85 (0.09-17.7)	74-fold	Current study
60/95 (63)	0.6 (0.3-9.2) a, *c*	fetal (fetal-0.08)	28-fold	Current study

a Values for samples with detectable levels of CYP2B6 protein.

b Code et al., 1997, Ekins et al., 1998, Stresser et al., 1999, Gervot et al., 1999

c Without a 36pmol/mg outlier.

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GENERAL DISCUSSION

There is an increasing awareness that CYP2B6 is an important xenobiotic metabolizing enzyme in humans that has been overlooked. The use of non-specific antibodies generated using non-human CYP2B proteins led to the erroneous belief that CYP2B6 was absent in most individuals, but antibodies used currently have revealed that almost all adults express CYP2B6 protein (Stresser and Kupfer, 1999). CYP2B6 is typically expressed at low-levels, but CYP2B6 is inducible and can become one of the main hepatic CYPs (Zhang et al., 2007). CYP2B6 is the principal metabolizing enzyme for several drugs and is also active towards a variety of environmental compounds (Hodgson and Rose, 2007). Risk assessment for regulatory purposes relies almost exclusively on data from surrogate animals, however surrogate animals cannot be used predict human variation, an essential component of risk assessment. Studies of human variation are consequently, an important adjunct to current risk assessment.

We identified CYP2B6 as the major CPO producing enzyme when using low substrate concentrations of CPS. CPO production decreased significantly when the substrate concentration dropped below 27μM, the Km of CPS desulfuration by CYP3A4. CYP2B6 activity was better correlated with CPO production than CYP3A4 activity when using lower concentrations of CPS. These results are in agreement with earlier work demonstrating high CYP2B6 activity using substrate concentrations of CPS below 5μM, and minimal CYP3A4 activity at these lower CPS concentrations (Foxenberg et al., 2007). Most CPS exposures are predicted to produce in vivo levels of less than 10μM CPS (Buratti et al., 2003). At these

concentrations, CYP2B6, a high affinity enzyme, with a Km for CPS desulfuration of 0.81μM, could be more significant than a more abundant low affinity enzyme such as CYP3A4. While CYP2B6 protein is frequently expressed in low amounts, CYP2B6 levels in the liver can vary over 350-fold and CYP2B6 is a major hepatic enzyme in some individuals (Hofmann et al., 2008)

The levels of CPO production determined in our studies was not be predicted by CYP2B6 genotype, but this lack of effect could be due to the small sample size (n=17). CYP2B6 activity for the phenotyped human liver microsomes used in the current study varied 274- fold (14-3840 pmol/mg*min), but did not differ by CYP2B6 genotype. The medical history of the individuals from whom these microsomal samples were obtained is unknown, and different exposures of the individuals to CYP2B6 inducers could explain much of the variability in CYP2B6 activity levels in these samples. While CYP2B6 protein levels have been reported to vary over 350-fold, from less than 1 pmol/mg to over 170pmol/mg, CYP2B6*6/*6 donors have been reported to have a lower range of CYP2B6 expression (Hoffmann et al., 2008). Adult liver samples from donors with the CYP2B6*1/*1 genotype have been reported to have CYP2B6 levels above 100pmol/mg but samples expressing the CYP2B6*6 allele have not (Hoffman et al., 2008). The CYP2B6 inhibitor ticlopidine reduced CPO production by samples with the CYP2B6*6/*6 genotype suggesting that the CYP2B6.6 protein is capable of metabolizing both CPS and ticlopidine.

CPO production varied more than 14-fold among individual human liver microsome samples using a substrate concentration of 20μM of CPS. One sample did not produce detectable levels of CPO and this sample also had the lowest levels of CYP2B6. Individuals

with higher levels of CYP2B6 activity produced the greatest amounts of CPO and could be at greater risk from CPS exposures, while individuals with lower CYP2B6 levels could be less susceptible to CPS poisoning.

We determined that CYP2B6 was developmentally regulated using human liver microsomes from 220 donors ranging in age from first-trimester to 17 years of age. Most samples expressed detectable levels of CYP2B6. This is in contrast to the findings of Tateishi et al. (1997), and the greater CYP2B6 expression found in the current study has implications for pediatric exposures to CYP2B6 substrates such as CPS. CYP2B6 levels were developmentally delayed and CYP2B6 protein expression increased significantly after the neonatal period. The percentage of samples with detectable CYP2B6 protein increased with age from 60% in the first trimester to 95% among samples from donors greater than 11 years of age.

An interaction between age and genotype was discovered in our analysis of CYP2B6 expression. The impact of developmental delays was masked by the presence of the CYP2B6*6 allele and only samples with the CYP2B6*1/*1 referent genotype had significant differences in CYP2B6 expression between age groups. CYP2B6 levels varied over 25-fold for fetal, infant, child and adolescent samples. Some prenatal and neonatal samples had CYP2B6 levels above the previously reported median levels of CYP2B6 protein found in adult livers (Hesse et al., 2004).

While PON1 and CYP2B6 levels have not been tested using the same donors, the regulation of the two genes differ and they are unlikely to follow similar expression patterns. PON1 is induced through sterol regulatory element-binding protein-2 (SREBP-2) activation,

while CYP2B6 is induced by CAR and PXR activation (Faucette et al., 2004). There are also differences in the ontogeny of PON1 and CYP2B6 expression. PON1 levels do not peak until at least six months of age and often not until two years of age (Furlong et al., 2005). In contrast, in the current study CYP2B6 levels showed a significant increase earlier in development and some fetal and neonate samples expressed CYP2B6 at levels above the reported median CYP2B6 levels for adults. Thus, it is possible that some children less than two years of age may have low PON1 levels, but adult levels of CYP2B6.

The levels of CPS detected in urban children in the United States have declined since the indoor ban was enacted (Williams et al., 2006), but agricultural use remains high and large quantities of CPS are manufactured in the United States (Stresser and Kupfer, 1999). Children can be exposed to pesticides through family members who are involved in the manufacture or use of CPS. The sale of CPS is largely restricted in the United States to licensed applicators, but CPS is more freely available in other parts of the world, where the use of CPS as a means of attempting suicide has been documented (Sebe et al., 2005).

CPS is of concern as a developmental toxicant (Sachana et al., 2008). One of the major protection mechanisms during pregnancy is the removal of toxicants by the transporter p-glycoprotein (pgp) in the placenta (Lankas et al., 1998). CPS is a poor human pgp substrate (Bain and LeBlanc, 1996) and there may be insufficient division between maternal and fetal exposures of CPS to protect the fetus from poisoning.

Chlorpyrifos has been found to cross the rodent placenta and has been linked to developmental delays (Levin et al., 2002). Chlorpyrifos can also cross the human placenta which leads to cholinergic toxicity and even fetal death *in utero* (Sebe et al., 2005).

Chlorprifos use has been documented in suicide attempts by pregnant women, and has resulted in fetal deaths due to fetal toxicity and CPS induced premature labor (Sebe et al., 2005; Solomon and Moodley, 2007).

An individual's CYP2B6 levels could significantly impact the risk associated with CPS exposure. CYP2B6 is capable of rapidly producing the neurotoxic metabolite CPO from low, physiologically relevant CPS levels. PON1 represents the main CPO metabolizing enzyme in humans and low PON1 levels could increase the risk of CPS poisoning. Future work in predicting susceptibility towards CPS poisoning should include information on both CYP expression and PON1 activity, ideally from the same donors.

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APPENDIX

Occupational Chlorpyrifos Exposure and Prostate Cancer Risk; the Role of CYP2B6, CYP2C19 and AhR polymorphisms.

E.L.Croom, S. Koutros, M.C. Alavanja and Ernest Hodgson.

ABSTRACT

The Agricultural Health Study is a large longitudinal prospective health study designed to follow thousands of licensed pesticide applicators working in North Carolina and Iowa. Early research in the Agricultural Health study reported an increased risk of developing prostate cancer among men with both a family history of prostate cancer and exposure to pesticides, including some organophosphate insecticides (Alavanja et al., 2003). DNA samples were collected from 533 participants in the Agricultural Health Study. 266 of the men were prostate cancer cases and 267 were matched controls. DNA samples were analyzed for the presence of polymorphisms in the metabolizing enzymes CYP2B6 and CYP2C19, and the aryl hydrocarbon receptor (AhR). Specifically, samples were assayed for the CYP2B6*6, CYP2C19*2 and the AhRG1661A polymorphisms. These are clinically relevant polymorphism. CYP2B6 and CYP2C19 metabolize organophosphate insecticides. The AhR is involved in carcinogenesis and a previous report linked this polymorphism to increased prostate cancer risk among pesticide applicators. Genotyping was performed on blinded samples using Taqman SNP Genotyping assays. Cases and controls did not differ significantly with respect to the presence of the CYP2B6*6, CYP2C19*2, or AhRG1661A alleles. Odds ratios were found to not be significant.

INTRODUCTION

Organophosphate insecticides (OPs) are widely used and moderately toxic compounds which act by inhibiting the enzyme AChE resulting in an increased level of actetycholine in the synapse and ultimately causing cholinergic toxicity.

Occupational exposure to certain OPs (fonofos, phorate, coumaphos, and chlorpyrifos) has been linked to an increased risk of developing prostate cancer. This increase only occurred among men with a family history of prostate cancer (Alavanja, 2003). OPs are bioactivated by cytochrome P450 (CYP) enzymes into their toxic oxon metabolites. Therefore we selected clinically relevant mutations in these OP metabolizing CYP genes.

The CYP2B6*6 allele has been linked to lower protein expression and altered metabolism of several CYP2B6 substrates. Patients with the *6/*6 genotype have been consistently found to have reduced metabolism towards the human immunodeficiency (HIV) reverse trancriptase drug efavirenz (Rotger et al., 2007). The CYP2C19*2 allele forms a splice variant that produces a truncated non-functional protein (Itoh et al., 1999). Individuals with the CYP2C19*2/*2 genotype have greatly reduced ability to metabolize drugs that are primarily metabolized by CYP2C19. In the case of warfarin, having just one CYP2C19*2 allele may reduce the ability to metabolize this anticoagulant drug (Itoh et al., 1999). In an earlier report (Aragaki et al., 2005) the AhRG1661A single nucleotide polymorphism (SNP) was linked to increased risk of prostate cancer linked to pesticide exposure. This SNP has also been associated with reduced survival time in patients with soft-tissue sarcomas (Berwick et al., 2004). The AhR is responsible for the ligand mediated induction of members

of the CYP1A family. CYP1A2 has been shown to be involved in the metabolism of several OPs in studies involving human liver microsomes (Tang et al., 2001; Buratti et al., 2003). The presence of the AHRG1661A SNP has also been linked to increased DNA damage among coke-oven workers (Chen et al., 2006).

MATERIALS AND METHODS

Materials. Taqman SNP Genotyping assays; C_25986767_70 (CYP2C19*2, rs4244285), C_7817765_60 (CYP2B6*6, rs3745274) and C__11170747_20 (AhR 1661G>A, rs2066853) and Taqman SNP Genotyping Master Mix were supplied by Applied Biosystems (Foster City, CA). An AhR 1661G>A positive DNA sample NA18564 was purchased from the Coriell Institute (Camden, NJ). All other reagents, if not specified, were purchased from commercial sources at the highest grade available.

Genotyping. Buccal cells were collected from Agricultural Health Study participants using mouthwash kits. DNA was extracted from buccal cells using commercially available kits. Samples from 270 individuals were processed by Whole Genome Amplification before genotyping. DNA was diluted to a concentration of 3ng/µl. Taqman SNP genotyping assays were used according to manufacturer's instructions (Applied Biosystems). DNA-free negative controls and previously genotyped positive controls were used for each polymerase chain reaction (PCR) analysis. Reagents were assembled in a PCR-dedicated hood. Reactions were run on an Eppendorf Mastercycler (Westbury, NY) and a post-reaction read was performed on a 7300 RT PCR system (Applied Biosystems).

Statistical Analysis. Unconditional logistic regression adjusted for the matching factor, age at enrollment, was used to quantify odds ratios (OR's) and 95% confidence intervals (95% CIs) for the association between a given polymorphism and prostate cancer. Genotypes were parameterized as ordinal variables 0,1,2 for analysis where 0=homozygote wild-type,

1=heterozygote, and 2=homozygote variant. Each polymorphism was evaluated under different genetic models; codominant, dominant, and recessive. Statistical tests were two-sided with an alpha-level of 0.05. All statistical analyses were performed using Stata version 9.0.

RESULTS

The frequencies of the AhR, CYP2B6 and CYP2C19 SNPs did not differ significantly between cases and controls (Table 1). For each assay 26 blinded duplicate quality control samples were included. Genotyping concordance was 96% for AhR 1661G>A, 92% for CYP2B6*6 and 88% for CYP2C19*2 assays. All three SNPs were in Hardy-Weinberg equilibrium. The odds ratios were not significant for any of the SNPs using dominant, codominant and recessive models (Table 2).

DISCUSSION

These three SNPs were not associated with a significant risk towards developing prostate cancer after pesticide exposure. This lack of significant findings does not necessarily mean that these SNPs play no role in pesticide metabolism or carcinogenesis, but their impact might be overcome by other factors. OP metabolism is complex, involving multiple activating and detoxifying enzymes. CYPs are responsible for the activation of chlorpyrifos and other OPs (Tang et al., 2001). Which CYP isoforms are most important is substrate specific (Buratti et al., 2003). CYP isoforms are also inducible and differences in CYP activity due differences in exposures to CYP inducers are possible. Those differences in enzyme activity cannot be determined by genotyping.

Several esterases detoxify OPs including carboxylesterases, butylesterases and paroxonases (Furlong et al., 2005). Paraoxonase 1 (PON1) has been well studied and is both inducible and polymorphic (Furlong et al., 2005). Other researchers plan to research the

possible impacts of PON1 status on the health status of the participants in the Agricultural Health Study.

In the future our research group plans to test in vitro the ability of the polymorphic CYP isoforms (CYP3A5.1, CYP2C9.3) and human liver microsomes with the genotypes CYP2B6*6/*6 to metabolize the OP chlorpyrifos. Larger studies are planned in the future that will look at more SNPs in other genes. This will allow for greater haplotype analysis.

Earlier dosimetry studies in the Agricultural Health Study assessed pesticide exposure levels and quantified urinary pesticide metabolite levels. DNA samples should be collected from the individuals that participated in the dosimetry and these samples should be genotyped. This would allow the comparison of urinary pesticide metabolite levels among individuals with different genotypes and known pesticide exposure levels.

Table 1. **Characteristics of Pesticide Applicators by case-control status in the Agricultural Health Study, 1993-2004**

Characteristic	rsnumber	Amino Acid Change	Cases n(%)	Controls n(%)
Total			266	267
Age at Enrollment (mean)	~	~	61.5	61.8
AHR-01	rs2066853	R554K		
GG			218 (82.0)	208 (77.9)
AG			44 (16.5)	55 (20.6)
AA			4 (1.5)	4 (1.5)
CYP2C19-03	rs4244285	P227P		
GG			196 (73.7)	183 (68.5)
AG			61 (22.9)	75 (28.1)
AA			9 (3.4)	9 (3.4)
CYP2B6-13	rs3745274	Q172H		
GG			159 (59.8)	149 (55.8)
GT			88 (33.1)	97 (36.3)
TT			19 (7.1)	21 (7.9)

Table 2. **Odds Ratios for selected polymorphims and risk of prostate cancer in the Agricultural Health Study, 1993-2004**
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Polymorphism	Case N=266	Control N=267	OR	95% CI
AHR-01				
Codominant Model				
GG	218	208	ref	~
AG	44	55	0.77	0.49, 1.19
AA	4	4	0.96	0.24, 3.90
Dominant Model				
GG	218	208	ref	
AA+AG	48	59	0.78	0.51, 1.19
Recessive Model				
GG + AG	262	263	ref	
AA	4	4	1.01	0.25, 4.09
CYP2C19-03				
Codominant Model				
GG	196	183	ref	
AG	61	75	0.76	0.51, 1.13
AA	9	9	0.92	0.34, 2.38
Dominant Model				
GG	196	183	ref	
AA+AG	70	84	0.78	0.53, 1.13
Recessive Model				
GG + AG	257	258	ref	
AA	9	9	0.99	0.39, 2.55
CYP2B6-13				
Codominant Model				
GG	159	149	ref	
GT	88	97	0.85	0.59, 1.23
TT	19	21	0.85	0.44, 1.65
Dominant Model				
GG	159	149	ref	
GT+TT	107	118	0.85	0.60, 1.20
Recessive Model				
GG + GT	247	246	ref	
TT	19	21	0.90	0.47, 1.72

Abbreviations: OR (Odds Ratio); CI: Confidence Interval.
* Adjusted for age

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