THE ONTOGENY OF HEPATIC DETOXIFICATION IN CHILDREN:
IMPLICATIONS FOR PEDIATRIC NON-ALCOHOLIC FATTY LIVER DISEASE

A DISSERTATION SUBMITTED TO THE GRADUATE DIVISION OF THE UNIVERSITY OF HAWAI‘I AT MĀNOA IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

IN

BIOMEDICAL SCIENCES (TROPICAL MEDICINE))

AUGUST 2011

BY

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KEYWORDS: UDP-GLUCURONOSYLTRANSFERASE, CYTOCHROME P450, PHARMACOKINETICS, DETOXIFICATION, OBESITY
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GENERAL ABSTRACT

The research presented in this dissertation provides, for the first time, a description of the ontogeny of hepatic antioxidants in the pediatric liver as well as the ontogeny of hepatic detoxification in children. Herein, we have studied the entire hepatic antioxidant defense network (with the exception of nitric oxide synthase) as it develops throughout childhood. Results demonstrated highest hepatic antioxidant capacity in the neonate that declines throughout childhood to a nadir around the age of ten then subsequently rebounds to adult levels after adolescence. Children may be less protected between the ages of six and ten years of age due to decreased antioxidant status (Chapter 2).

Additionally, the ontogenies of four different UDP-glucuronosyltransferase isoforms (UGTs 1A1, 1A4, 1A6, and 1A9) were examined. Experiments showed each enzyme were independently regulated, maturing at different rates and ages. In general, these enzymes showed full activity by two years of age. Interestingly, UGT1A1, 1A4 and 1A6 maturation may be regulated post-translationally and/or allosterically while UGT1A9 matures mostly due to transcriptional effects. When scaled to whole liver, hepatic clearances matured later, from one to eighteen years of age. Furthermore, the age of maturation varied between the four clearance models (well-stirred and parallel tube models using allometric pharmacokinetics versus physiology-based pharmacokinetics). The difference indicated that one model cannot be applied to every isoform and must be evaluated on a case-by-case basis (Chapters 3 – 5). Since clearance matures years after birth, children may be more susceptible to chemical toxicities and subsequent liver damage. Coupled together, these descriptions provide a general status of hepatic detoxification capacity. Lack of this capacity, due to environmental or genetic effects,
may have implications in the role of some pediatric liver diseases, such as in pediatric non-alcoholic fatty liver disease (Chapter 6). Overall, these data presented herein have substantial value to the field of developmental pharmacology and pediatric pharmacokinetics, as well as potential reasons for drug toxicities, adverse drug reactions, and liver diseases in children.
DEDICATION

I dedicate this dissertation to my mother, Shigeko Miyagi, who is the most influential person in my life. Through her gentle wisdom, my mother taught me to pursue knowledge with passion and drive. Finally, I want to thank her for her endless support in my many academic pursuits (especially the time I almost dropped out of college to become a chef). Without her love and support, none of this would have been possible.
ACKNOWLEDGMENTS

I would like to acknowledge my advisor, Dr. Abby C. Collier, for her continued support, encouragement throughout this process, and the many opportunities for professional growth; my committee members, Drs. Vivek Nerurkar, Robert Nichols, Alexander Stokes, Diane Taylor, and Karen Thompson, for their assistance in the interpretation and analysis of my research; my fellow lab mates and graduate students who have been essential in the completion of my research, especially Zach Bergeron, Kristen Milam, Luc Rougée, Jeff Raunig, Brittany Sato, Ron Tamayo, and Audrey Thevenon; my family and friends for keeping me sane throughout this process; and finally, my best friend Francis Lam, who has supported me for the past 16 years, particularly during my trips to NYC.
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Equation 1.1  \[ BMI \ (kg \ / \ m^2) = \frac{Weight \ in \ kg}{Height \ in \ m^2} \]

Equation 3.1  \[ CL_{hepatic} (L/\text{hr}) = \frac{Q_{hepatic} \times f_u \times CL_{max}}{Q_{hepatic} + f_u \times CL_{max}} \]

Equation 3.2  \[ CL_{hepatic} (L/\text{hr}) = Q_{hepatic} \times \left(1 - e^{-\frac{CL_{max} \times f_u}{Q_{hepatic}}}ight) \]

Equation 3.3  \[ CL_{pediatric} (L/\text{hr}) = CL_{hepatic} \times \left(\frac{W_i}{W_{std}}\right)^\frac{3}{4} \]

Equation 3.4  \[ Y = Y_{max} \times (1-e^{-KX}) \]

Equation 3.5  \[ Y = Span \times e^{-KX} + plateau \]

Equation 4.1  \[ Y = Y_0 + (Y_{max} - Y_0)(1-e^{-KX}) \]

Equation 4.2  \[ Liver \ Size(g) = (Body \ Surface \ Area)^{1.176} \times 0.722 \]

Equation 4.3  \[ Q_{hepatic} (L/\text{hr}) = 0.265 \times 10^{(-0.6492 \times Age - 0.143 \times Age^2 + 0.08891 \times Age^3)} \]

Equation 4.4  \[ MPPGL \ (mg/g) = 10^{(1.407 + 0.0158 \times Age - 0.000382 \times Age^2 + 0.000024 \times Age^3)} \]

Equation 4.5  \[ [P]_{Pediatric} \ (g/L) = 1.1287 \times LN(Age) + 33.746 \]
Equation 4.6  \[ f_{u, \text{Pediatric}} = \frac{1}{1 + \frac{(1 - f_{u, \text{Adult}}) \times [P]_{\text{Pediatric}}}{[P]_{\text{Adult}} \times f_{u, \text{Adult}}}} \]

Equation 4.7  \[ \text{Body surface area (m}^2 \text{)} = 0.007184 \times \text{Height}^{0.725} \times \text{Weight}^{0.425} \]

Equation 5.1  \[ v = \frac{V_{\text{max}} \times [S]}{K_m (1 + [I] / K_i) + [S](1 + [I] / K_i)} \]

Equation 5.2  \[ CL_{\text{max}} = \frac{V_{\text{max}} \times (n-1)}{S_{50} n(n-1)^a} \]
**LIST OF ABBREVIATIONS**

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<tr>
<td>[I]</td>
<td>inhibitor concentration</td>
</tr>
<tr>
<td>[P]_{adult}</td>
<td>amount of adult albumin</td>
</tr>
<tr>
<td>[P]_{pediatric}</td>
<td>amount of pediatric albumin</td>
</tr>
<tr>
<td>[S]</td>
<td>substrate concentration</td>
</tr>
<tr>
<td>°C</td>
<td>degrees Celsius</td>
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<tr>
<td>µg</td>
<td>microgram</td>
</tr>
<tr>
<td>µM</td>
<td>micromoles per liter</td>
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<tr>
<td>1H-NMR</td>
<td>proton nuclear magnetic resonance</td>
</tr>
<tr>
<td>4MU</td>
<td>4-methylumbelliferone sodium salt</td>
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<td>4MUG</td>
<td>4-methyl umbelliferone glucuronide</td>
</tr>
<tr>
<td>6PGδL</td>
<td>6-phosphoglucono-delta-lactone</td>
</tr>
<tr>
<td>A</td>
<td>an oxidized substrate</td>
</tr>
<tr>
<td>AH_2</td>
<td>a reduced substrate</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>Arg</td>
<td>arginine</td>
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<tr>
<td>ASBT</td>
<td>apical sodium-dependent bile acid transporter</td>
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<tr>
<td>ASC</td>
<td>ascorbate</td>
</tr>
<tr>
<td>ASC•</td>
<td>semi-dehydroascorbate</td>
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<td>BLAST</td>
<td>basic local alignment search tool</td>
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<td>BMI</td>
<td>body mass index</td>
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<td>BSA</td>
<td>body surface area</td>
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<td>BSEP</td>
<td>bile salt export pump</td>
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<tr>
<td>C</td>
<td>control (no fat loading)</td>
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<tr>
<td>CAT</td>
<td>catalase</td>
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<td>cGST</td>
<td>cytosolic glutathione-S-transferase</td>
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<td>CHO</td>
<td>Chinese hamster ovary cell line</td>
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<tr>
<td>CI</td>
<td>confidence interval</td>
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<tr>
<td>Cit</td>
<td>citrulline</td>
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<td>CL_{hepatic}</td>
<td>hepatic clearance</td>
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<td>CL_{int}</td>
<td>intrinsic clearance</td>
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<tr>
<td>CL_{max}</td>
<td>max clearance</td>
</tr>
<tr>
<td>CL_{pediatric}</td>
<td>pediatric hepatic clearance</td>
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<tr>
<td>ClustalW</td>
<td>multiple sequence alignment program, version omega</td>
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<tr>
<td>cm</td>
<td>centimeter</td>
</tr>
<tr>
<td>Cu</td>
<td>copper</td>
</tr>
<tr>
<td>Cu^{2+}</td>
<td>copper (II) ion</td>
</tr>
<tr>
<td>CV</td>
<td>coefficient of variance (standard deviation divided by the mean)</td>
</tr>
<tr>
<td>CYP</td>
<td>cytochrome P450 super-family</td>
</tr>
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<td>CYP1A</td>
<td>cytochrome P450 1A subfamily</td>
</tr>
<tr>
<td>CYP1A1</td>
<td>cytochrome P450 1A1 isoform</td>
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<td>CYP2C</td>
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<tr>
<td>CYP2C8</td>
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<tr>
<td>Symbol</td>
<td>Description</td>
</tr>
<tr>
<td>--------</td>
<td>------------------------------------------</td>
</tr>
<tr>
<td>K</td>
<td>rate of development</td>
</tr>
<tr>
<td>kDa</td>
<td>kilodalton</td>
</tr>
<tr>
<td>kg</td>
<td>kilogram</td>
</tr>
<tr>
<td>$K_i$</td>
<td>inhibition constant</td>
</tr>
<tr>
<td>$K_{ma}$</td>
<td>Michaelis-Menten constant</td>
</tr>
<tr>
<td>L</td>
<td>liter</td>
</tr>
<tr>
<td>$L^\cdot$</td>
<td>a lipid radical</td>
</tr>
<tr>
<td>LH</td>
<td>a lipid</td>
</tr>
<tr>
<td>LOH</td>
<td>an hydroxylated lipid</td>
</tr>
<tr>
<td>$LOO^\cdot$</td>
<td>a lipid peroxyradical</td>
</tr>
<tr>
<td>LOOH</td>
<td>a lipid hydroperoxide</td>
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<tr>
<td>M</td>
<td>moles per liter</td>
</tr>
<tr>
<td>mg</td>
<td>milligram</td>
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<td>MgCl$_2$</td>
<td>magnesium chloride</td>
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<td>mGST</td>
<td>microsomal glutathione-S-transferase</td>
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<td>millimoles per liter</td>
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<tr>
<td>MO</td>
<td>morbidly obese</td>
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<td>moles</td>
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<td>MPPGL</td>
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<td>NADPH</td>
<td>alpha-nicotinamide adenine dinucleotide phosphate (reduced)</td>
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<td>non-alcoholic fatty liver disease</td>
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<td>sodium hydroxide</td>
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<td>NAT</td>
<td>N-acetyltransferase</td>
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<tr>
<td>NCBI</td>
<td>National center for Biotechnology Information</td>
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<tr>
<td>NFA</td>
<td>niflumic acid</td>
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<td>NF-κB</td>
<td>nuclear factor kappa-light-chain-enhancer of activated B cells</td>
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<tr>
<td>ng</td>
<td>nanograms</td>
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<td>NHANES</td>
<td>National Health and Nutrition Examination Survey</td>
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<td>NIH</td>
<td>National Institutes of Health</td>
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<tr>
<td>nm</td>
<td>nanometer</td>
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<td>nM</td>
<td>nanomoles per liter</td>
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<tr>
<td>NO$^\cdot$</td>
<td>nitric oxide</td>
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<tr>
<td>NOS</td>
<td>nitric oxide synthase</td>
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<td>NQO1</td>
<td>NADPH dehydrogenase, quinine 1</td>
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<td>NTCP</td>
<td>Na$^+$ taurocholate cotransporting polypeptide</td>
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<td>NW</td>
<td>normal weight</td>
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<tr>
<td>OB</td>
<td>obese</td>
</tr>
<tr>
<td>OST</td>
<td>organic solute transporter</td>
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</table>
OSTα  organic solute transporter isoform alpha
OSTβ  organic solute transporter isoform beta
OW  overweight
PAPS  3′-phosphoadenosine-5′-phosphosulfate
PBPK  population-based pharmacokinetics
PBS  phosphate buffered saline
PBS-T  phosphate buffered saline with Tween 20
PK  pharmacokinetics
pmol  picomoles
PPAR  peroxisomal proliferation activator receptor
PPARα  peroxisomal proliferation activator receptor isoform alpha
PPARγ  peroxisomal proliferation activator receptor isoform gamma
PPARδ  peroxisomal proliferation activator receptor isoform delta
PS  photosensitizer
PVDF  polyvinylidene difluoride
Q  ubiquinone
QH•  ubisemiquinone
QH2  ubiquinol
Qhepatic  hepatic blood flow
RNA  ribonucleic acid
ROS  reactive oxygen species
ROUT  robust non-linear regression and outlier removal
S50  half-maximum of Vmax
S9  post-mitochondrial sub-cellular fraction
SD  standard deviation
SDS-PAGE  sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM  standard error of mean
SOD  superoxide dismutate
SULT  sulfotransferase superfamily
SULT1  sulfotransferase 2 subfamily
SULT1A1  sulfotransferase 1A1 isoform
SULT1E1  sulfotransferase 1E1 isoform
SULT2  sulfotransferase 1 subfamily
SULT2A1  sulfotransferase 2A1 isoform
Sy.x  standard error of estimates
TBS  Tris-buffered saline
TBS-T  Tris-buffered saline with Tween-20
TFP  trifluoperazine
TG  triglycerides
TMB  3,3′,5,5′-Tetramethylbenzidine
TNF  tumor necrosis factor
TOC  alpha-tocopherol
TOC•  alpha-tocopheryl radical
TR  thioredoxin reductase
Tris  tris(hydroxymethyl)aminomethane
Trx  thioredoxin reductase
UDP uridine diphosphate
UDPGA UDP-glucuronic acid
UGT UDP-glucuronosyltransferase super-family
UGT1A UDP-glucuronosyltransferase 1A sub-family
UGT1A1 UDP-glucuronosyltransferase 1A1 isoform
UGT1A10 UDP-glucuronosyltransferase 1A10 isoform
UGT1A3 UDP-glucuronosyltransferase 1A3 isoform
UGT1A4 UDP-glucuronosyltransferase 1A4 isoform
UGT1A6 UDP-glucuronosyltransferase 1A6 isoform
UGT1A7 UDP-glucuronosyltransferase 1A7 isoform
UGT1A8 UDP-glucuronosyltransferase 1A8 isoform
UGT1A9 UDP-glucuronosyltransferase 1A9 isoform
UGT2A UDP-glucuronosyltransferase 2A sub-family
UGT2B UDP-glucuronosyltransferase 2B sub-family
UGT2B15 UDP-glucuronosyltransferase 2B15 isoform
UGT2B7 UDP-glucuronosyltransferase 2B7 isoform
UNOS United Network for Organ Sharing
V_{max} maximum reaction velocity
W_i individual weight
W_{std} standard average weight
Y_0 initial activity after birth
Y_{max} max enzyme rate
Zn zinc
\varepsilon extinction coefficient
\lambda wavelength
PAPERS ARISING FROM THIS DISSERTATION

Published papers


Papers submitted for publication


Papers in preparation


xxi
Conference abstracts


CHAPTER 1

GENERAL INTRODUCTION
INTRODUCTION

Pediatric nonalcoholic fatty liver disease (NAFLD) is one of the most significant childhood health problems in the developed world (Patton et al., 2006). However, the exact causes of this disease are unknown. Recognized in 1980, NAFLD is a clinical term covering a wide range of liver diseases from simple steatosis (hepatic lipid accumulation without inflammation) to non-alcoholic steatohepatitis (NASH, hepatic lipid accumulation with inflammation and fibrosis) to cirrhosis, all in the absence of alcohol (Xanthakos et al., 2006). The prevalence of NAFLD is not well described (Lerret and Skelton, 2008), but severest cases of pediatric NAFLD occurs almost exclusively in the 10 to 14 year age range, with twice as many boys as girls and is highly correlated with obesity and diabetes (Lavine and Schwimmer, 2004).

The pathogenesis of NAFLD and its progressive diseases remain unclear, but researchers have suggested a “two-hit” hypothesis. First, hepatic lipid accumulation causes increases in reactive oxygen species (ROS). Second, increased ROS overpowers hepatic antioxidant capacity, leading to the release of inflammatory cytokines, fibrosis and eventually NASH (Reddy and Rao, 2006). Previous studies show important hepatic detoxification and antioxidant enzymes can be inhibited by free fatty acids (FFAs) (Mitra et al., 1992; Singh et al., 1988; Schneider, 1992; Schneider et al., 1993; Tsoutsikos et al., 2004). Considering these hepatic detoxification enzymes also metabolize ROS generating compounds, such as bile acids (Barbier et al., 2009; Baijal et al., 1998; Hayes and Mantle, 1986), it is possible that the buildup of hepatic FFAs inhibit detoxification, such as bile acid metabolism, leading to increased levels of ROS that will, in turn, damage the liver.
Susceptibility to liver damage, and hence NAFLD, may be increased during 10 to 14 years of age because of reduced hepatic enzyme detoxification capacity in this age rage.

The aim of this study is to investigate molecular basis of pediatric NAFLD by examining the interplay of developmental (ontogeny of hepatic antioxidant capacity and detoxification enzymes) and environmental (obesity, a syndrome generally caused through excessive calorie intake and inadequate exercise) factors in pediatric and adult liver tissues. There are 3 major hypotheses in this dissertation:

1) We hypothesize that children have inadequate antioxidant capacity, which predisposes them to NAFLD.

2) We hypothesize that UDP-glucuronosyltransferase (UGT) isoforms essential for bile acid, ROS, hormone and chemical detoxification take months-to-years to develop in the neonatal liver, as does the clearance capacity in hepatic UGT-mediated pathways.

3) We hypothesize that obesity causes inhibition of liver metabolism and detoxification through the effects of FFAs.

By investigating these hypotheses, we hope to identify critical developmental aspects of NAFLD pathogenesis. These developmental mechanisms can be targeted for pharmacological and medical interventions. An additional outcome will be to assist in the
rational design of drug dosage regimens in children based on knowledge of pediatric pharmacology. Finally, by determining the inhibitory effects of FFAs on hepatic detoxification enzymes, we hope to identify a critical point in the etiology of obesity-associated morbidity that can be targeted for symptomatic and disease-specific relief.

**DRUG METABOLISM AND BIOTRANSFORMATION**

The human body is exposed to many xenobiotics, or foreign chemical compounds, throughout their lifetime. These “foreign compounds” comprise industrial, agricultural, environmental and dietary sources, and as such, it is important to limit human exposure to these chemicals. However, where exposure cannot be minimized, it falls largely to the liver to detoxify parent compounds and reactive intermediates from these exposures. Adults can efficiently and effectively remove many xenobiotics, preventing damage (Correia, 2009). This process is known as drug metabolism, or drug biotransformation. While many organs, such as the intestines and kidneys, can metabolize drugs, the main site for biotransformation occurs in the liver, primarily in hepatocytes (Miners, 2002). The intracellular localization of drug metabolizing enzymes is the membrane of the endoplasmic reticulum, although some significant enzymes are also found in the cytosol of the cell (Miners, 2002).

Drug metabolism can be divided into two major categories: Phase I and Phase II. Phase I reactions involve the reduction or oxidation of a compound, which typically introduces or unmasks a functional group, such as \(-\text{OH}, \ -\text{SH} \text{ or } \ -\text{NH}_2\). Phase II reactions are categorized as conjugation reactions, meaning these enzymes add large polar moieties to these
compounds to help the body eliminate the xenobiotic through the urine or feces. Compounds can either undergo Phase I, Phase II, or both Phase I and II reactions before the substance is eliminated (Correia, 2009).

The majority of Phase I reactions are carried out by the superfamily of enzymes known as cytochrome P450s (CYPs). The CYPs require both a reducing agent (NADPH) and molecular oxygen and are found in the endoplasmic reticulum. First, the NADPH-cytochrome P450 reductase uses the cofactor NADPH to transfer an electron to the cytochrome P450 complex. Using molecular oxygen and the donated electron, the protein is able to oxidize the xenobiotic. This oxidation allows the opening or introduction of functional groups; these groups make the compound more lipophobic and also allow further biotransformation by the Phase II enzymes. While the CYPs carry out more than 80% of the Phase I reactions, other enzymes in this category include the flavin-containing monooxygenases, epoxide hydrolases and esterases (Correia, 2009).

Phase II reactions are primarily conjugation reactions, with the superfamily UDP-glucuronosyltransferases (UGTs) accounting for more than one-third of drug metabolism activity (Correia, 2009). Most Phase II reactions add a large polar group to the xenobiotic that makes the compound substantially more hydrophilic. In particular, UGTs, like the CYPs, are found in the endoplasmic reticulum and use the cofactor UDP-glucuronic acid (UDPGA). Other Phase II enzymes include the sulfotransferases (SULT), the glutathione-S-transferase (GST) and N-acetyltransferases (NAT). Like the UGTs, these other enzymes conjugate the xenobiotics using their obligate cofactors and increase
hydrophilicity. However, these enzymes are found in the cytosol (Correia, 2009).

Many drug metabolizing enzymes are super-families, meaning that multiple isoforms are
produced from a common ancestral gene (Glasner et al., 2006). The ability of each
isoform to metabolize multiple compounds is the hallmark of drug metabolism, since
metabolic pathways generally include wide redundancy. Specifically, the xenobiotic
metabolizing enzymes have high affinity and wide-substrate specificity; which is the
biochemical basis for wide levels of redundancy in drug, chemical and hormone
biotransformation capability. When these enzymes are at full capacity, they provide the
ability to protect the body from toxic substances. While these enzymes are primarily used
to rid the body of xenobiotics, they are also involved in the normal metabolism of dietary
and endogenous compounds, such as hormones, neurotransmitters and bile acids
(Radominska-Pandya et al., 1999; Zhang and Yang, 2009; Barbier et al., 2009).

While these statements typically hold true in adults, they may not be true for children.
When the body cannot adequately detoxify reactive chemicals and/or when metabolism
produces reactive metabolites, negative biological consequences may occur (Pirmohamed
and Park, 2003). Adverse drug reactions are included in these “negative biological
consequences” and are one of the leading causes of pediatric death and illness in the US
(Impicciatore et al., 2001), leading to approximately 79,000 pediatric hospitalizations per
year, 31,000 of which are life threatening or fatal (Miller et al., 2003). The liver’s
capacity for drug metabolism and elimination in children is very different from adults.
There are few studies on the development of drug metabolism in children and only
marginally greater number of studies reporting on safe dosing of drugs in children
(Strolin Benedetti et al., 2005). Hence, most pediatric formularies have come into use through trial-and-error on the part of experienced physicians. For example, acetaminophen has a longer half-life in children less than two years of age than in adults, which is why there are different dosages for neonates (0 – 28 days), infants (1 – 12 months), children (1 to 12 years), adolescents (13 – 19 years) and adults (van der Marel et al., 2003). Despite the lack of evidence-based trials, it is widely believed that the main cause of adverse drug reactions in children is due to a developmental lack of drug metabolizing capabilities.

Medications that have not been adequately studied for safety in pediatric medicine are being prescribed more to children. For example, a recent study of pediatric wards in Finland showed that 76% of the patients had at least one off-label or unlicensed drug prescribed, with 79% of those patients in the neonatal intensive care unit (Lindell-Osuagwu et al., 2009). Prescribing off-label can lead to pediatric adverse drug reactions. These types of reviews underline the lack of controlled studies to support evidence-based practice in clinical pediatrics. Therefore, it is important to focus on pediatric pharmacology as it will provide a beginning for relevant framework to understand and integrate developmental physiology and pharmacology. This will help to understand childhood development, inform drug use and development and improve pediatric clinical medicine.

THE ONTOGENY OF CYTOCHROMES P450

The developmental dynamics of the human cytochrome P450 (CYP) superfamily of
enzymes (EC 1.14.14.1) are comparatively well-studied in developmental pharmacology in regards to the other drug metabolizing enzymes. However, despite the vast focus on CYP research in the 30 – 40 years, ontogeny data is still sparse. Divided into 18 families and 42 subfamilies, the CYPs are responsible for the majority of biotransformation reactions, specifically only 23 enzymes belonging to families one through three are considered clinically relevant (Hines, 2008), while 90% of drug metabolism is performed by only five isoforms. Apart from these five, CYP enzymes are generally involved in the synthesis and metabolism of endobiotics (Guengerich, 2006).

Table 1.1. Data on CYP ontogeny.

<table>
<thead>
<tr>
<th>CYP Ontogeny</th>
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<td>1A1</td>
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<td>2C8</td>
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<td>3A7</td>
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The CYP1A family, comprising of CYP1A1 and 1A2 isoforms, is primarily responsible for the biotransformation of polycyclic aromatic hydrocarbons. CYP1A1 is not constitutively expressed in the human liver, and is only activated by xenobiotics exposure, such as with cigarette smoke and car exhaust fumes (McLemore et al., 1990). It has been shown that CYP1A1 is constitutively expressed during organogenesis, but declines to non-detectable levels afterwards (Shao et al., 2007). Furthermore, although constitutively present in adult liver, CYP1A2 is not detectable in fetal tissue (Carrier et al., 1988) but is
detectable in neonatal tissues, with expression levels immediately after birth ten times lower than in adults (Blake et al., 2006).

The CYP2C family is responsible for the oxidative metabolism of approximately 30% of clinically relevant drugs (Williams et al., 2004). These isoforms include CYP2C8, CYP2C9, CYP2C18, and CYP2C19. The ontogeny of CYP2C8 and CYP2C18 is not known (Hines, 2008). Koukouritaki et al. (2004) demonstrated that CYP2C9 was detectable between 8 weeks and 24 weeks of gestation, albeit at 1% of adult levels. Activity rapidly increases between 25 weeks and 40 weeks of gestation to 10% of adult levels and then to 25% by 5 months of age. Interestingly, CYP2C9 seemed to not reach adult levels even after puberty. The CYP2C19 isoform reaches 10 - 20% of adult levels by 40 weeks of gestation and increases to 75% of adult activity by 5 months of age.

The isoform CYP2D6 is responsible for the oxidative metabolism of approximately 12% of clinically relevant drugs (Williams et al., 2004). Like the CYP1A2, CYP2D6 is not expressed until approximately 30 weeks of gestation. At birth, there is detectable activity but at 5% of adult levels. This steadily increased to 30% of adult activity by 28 days of age. By 5 years of age, that activity rose to 70% of adult levels (Treluyer et al., 1991).

Although CYP2E1 makes up approximately 7% of total hepatic CYP content, the isoform only accounts for 2.5% of clinically relevant drug metabolism in adults. Despite this, it is vital in the metabolism of several important drugs, including acetaminophen and halothane (Williams et al., 2004). Viera et al. (1996) demonstrated that CYP2E1 was
undetectable in fetal tissue, but was approximately 10% of adult activity in newborns. By 10 years of age, the activity of CYP2E1 is approximately the same as adults. Johnsrud et al. (2003) further investigated the expression during gestation and found that hepatic CYP2E1 is expressed by the late second or third trimester, but they could not confirm precise age of maturity, locating it somewhere between 3 months and 18 years of age.

The CYP3A family is the most important drug metabolizing class and accounts for more than 45% of the metabolism for clinically relevant drugs. It consists of CYP3A4, CYP3A5, CYP3A7 and CYP3A43. The CYP3A4 isoform is the most active and commonly described as the single most important drug-metabolizing enzyme (Williams et al., 2004). Due to its importance, many studies have assessed the ontogeny of the CYP3A family. It seems that CYP3A4 and CYP3A7 are present at approximately 10% of adult activity in the fetal liver (<30 weeks gestational age). Interestingly, CYP3A7 matures faster than CYP3A4 after birth, becoming the dominant CYP3A isoform until around the child’s first birthday, when CYP3A4 increases to become the dominant isoform as CYP3A7 declines (Lacroix et al., 1997).

While CYPs metabolize the majority of drugs, it important to note that these enzymes work much slower than their Phase II counterparts, approximately 100 to 1000 fold less (i.e. pmol/min/mg protein versus nmol/min/mg protein). Since these works at a much greater capacity, it is also important to know ontogeny of Phase II enzymes as well.
THE ONTOGENY OF UDP-GLUCURONOSYLTRANSFERASES AND OTHER PHASE II ENZYMES

After the CYPs, the UGTs (E.C. 2.4.1.17) are one of the most critical families for clearance and elimination of drugs. They are also vital for metabolism and clearance of endobiotics, dietary compounds, environmental chemicals, and hormones, making them considerably broader in function than the CYPs and, perhaps, of equal or greater importance. The UGTs account for almost 15% of drug clearance in the human body (Williams et al., 2004). Despite the importance of these enzymes, published data on their developmental dynamics scarcely exist (Radomsinska-Pandya et al., 1999).

In humans, there are three subfamilies of UGT enzymes: designated UGT1A, UGT2A and UGT2B. The UGT2A family is only found in the nasal mucosa and is involved in smell; hence, these UGT isoforms will not be discussed further (Kiang et al., 2005). While it is well-recognized that the majority of UGTs are constitutively expressed in adult human liver, the ontogeny of UGTs is relatively undefined. This area of research has been hampered, until recently, by a lack of specific probe substrates or inhibitors of UGTs (Court, 2005).

With respect to development, it is interesting that UGT activity is largely absent from the fetal liver (Collier et al., 2002a; Collier et al., 2002b). In the early 1980s, a late-fetal change (30 – 40 weeks of gestation) in hepatic UGT1A1 (from 0.1% to 1.0% of adult activity levels) and post-natal changes that are related to birth age, not gestational age, were reported (Kawade and Onishi, 1981). Subsequently, researchers used biochemical
substrates to determine general UGT activity in the fetal liver, demonstrating that bilirubin (UGT1A1) and 1-naphthol (UGT1A1, UGT1A3, UGT1A6, UGT1A7, UGT1A8, UGT1A9, UGT1A10, and UGT2B7) glucuronidation increase in the post-natal period. Despite these activity differences, similar levels of protein expression were observed between a 13-week neonate and an adult liver. Since neither the antibody used in this assay nor the substrates were specific, evidence for the maturation of single isoforms was not inferred (Coughtrie et al., 1988). Following this, Burchell et al. (1989) demonstrated UGT maturation with fetal and neonatal liver samples from several gestational time points. Similar development of general UGT activities to different substrates were observed except for serotonin (UGT1A4), where adult activities were observed in fetal (16 – 25 weeks) and neonatal liver up to 10 days old. More recently, individual UGT isoform development in infants and young children, including two fetal liver samples, were analyzed and showed that pediatric levels of mRNA and protein for UGT isoforms did not differ from adults, but activities were lower up to 2 years of age (Strassburg et al., 2002). Finally, Zaya et al. (2006) demonstrated the maturation of UGT2B7. Like previous UGT enzymes, they showed a progressive increase in activity from birth to 18 years of age. Unlike the other UGT studies, UGT2B7 showed a correlation between protein expression and activity.

Another major biotransformation and clearance family are the GST enzymes (EC 2.5.1.18). The GSTs conjugate xenobiotics to glutathione, the obligate cofactor for GST, to form mercapturic acids. Glutathione also serves as an antioxidant in the liver, and when there is increased oxidative stress in the liver, the supply of GSH also dwindles,
which leads to decreased GST capacity in the liver. There are three different types of GSTs: cytosolic, microsomal, and mitochondrial. Cytosolic GSTs are the most important for drug metabolism, while the other two are involved in the synthesis and regulation of endobiotics such as prostaglandins and ecosanoids (Hayes et al., 2005). The ontogeny of GST is less defined as than UGTs and CYPs. There are over 16 different GST isoforms within six subfamilies. While there are few gene-specific developmental patterns defined, it appears that the cytosolic GST family is expressed at high levels during gestation and after birth, suggesting an important role in development. Although the exact role in this is not well understood, high neonatal oxidative stress can lead to retinopathy of prematurity (Saugstad, 2005). Furthermore, null or mutant polymorphisms in certain GSTs may be a risk factor for age-related macular degeneration (Oz et al., 2006). Together, this may indicate the protective role of GSTs in early development.

Lastly, the sulfotransferases (SULTs, EC 2.8.2.1) are another superfamily of soluble enzymes that conjugate xenobiotics using 3’-phosphoadenosine-5’-phosphosulfate (PAPS). Interestingly, sulfonates are not produced in the neonate. Instead these precursors to PAPS are obtained from maternal circulation. The SULT enzymes mediate sulfonation of numerous endogenous compounds, which may bioactivate or eliminate them. Therefore the role(s) of SULT in early development are vital (Dawson, 2011). There are three SULT families, of which the SULT1 and SULT2 are the most important in drug metabolism (Hines, 2008). Duanmu et al. (2006) demonstrated various ontogenetic profiles for the SULT1A1, SULT1E1 and SULT2A1. Interestingly, SULT1A1 shows no change in the fetal, perinatal and postnatal liver. However,
SULT2A1 showed detectable levels in the fetal liver but increased significantly after birth, achieving adult levels after 3 months of age. SULT1E1 was in stark contrast, showing highest activity in the fetal liver and decreasing to its lowest activity after 3 months of age.

Data describing the ontogeny of drug metabolism are scarce. Evidence thus far demonstrates that not all enzymes develop at the same rate or extent. Some are present in both fetal and adult tissue; others only become functional after birth and the remaining few metabolizing enzymes are most active in fetal tissue and decrease after birth (Hines, 2008). Additionally, several enzymes have been studied at discrete time points or in specific diseases, but in general their rates of maturation have not been defined. Since it has been inferred (for example from changes required in drug dosing) that many of these enzymes work at decreased capacity in neonates and children, it is important to understand the development of enzyme capacity and systemic detoxification. This supports the previous assertions that decreased drug metabolism capacity in children is a possible cause of adverse drug reactions. For example, if adults have 10-fold detoxification capacity compared to children, levels of enzyme inhibition that are unnoticeable in adults may be detrimental in children through accumulation of toxic products and ROS, leading to liver damage.

Of further interest, metabolizing enzymes are highly variable in the population and contain multiple functional polymorphisms. Therefore in children where enzyme activity is already developmentally low, the presence of functional polymorphisms that confer
lower enzyme activity may tip the child above the threshold for toxicity. Currently available data only provide a rough estimate of when drug metabolism matures and more precise knowledge of the ontogeny of individual enzymes is necessary for better understanding of developmental pharmacology, disease processes and drug administration.

**DRUG METABOLISM, OBESITY AND PEDIATRIC LIVER DISEASE**

Between 1980 and 2002, the prevalence of children classified as overweight has tripled and it is now estimated that 17% of US children are overweight (Ogden et al., 2006). Increased access to food and sedentary lifestyles are believed to be the main cause of this epidemic (Hussain and Bloom, 2011). Psychological and physiological problems arising from obesity include depression, degenerative joint disease, type 2 diabetes, hyperlipidemia, hypertension, and chronic renal dysfunction (Choudhary et al., 2007). Formally recognized in 1980, NAFLD has recently been added to this list of diseases. The term NAFLD encompasses a wide range of liver diseases; from simple steatosis (lipid droplet accumulation in the liver without associated inflammation) to NASH (lipid accumulation with inflammation and fibrosis of the liver) to cirrhosis, and all with the absence of alcohol. The disease can also progress to end-stage liver disease or cancer (Dunn and Schwimmer, 2008).

Currently, NAFLD is the most common pediatric liver disease, with incidence increasing at an alarming rate (Xanthakos et al., 2006). The prevalence of NAFLD is not well described (Lerret and Skelton, 2008), but the severest cases of pediatric NAFLD occurs
almost exclusively in the 10 to 14 year age range, with twice as many boys as girls and is highly correlated with obesity and diabetes (Lavine and Schwimmer, 2004). Pathogenesis of NAFLD and its progression remain unclear, but researchers have suggested a “two-hit” hypothesis. First, hepatic lipid accumulation causes increases in ROS. Second, increased ROS overpowers hepatic antioxidant capacity, leading to the release of inflammatory cytokines, fibrosis and eventually NASH (Figure 1.1) (Reddy and Rao, 2006).

Because previous studies have demonstrated that drug metabolizing and antioxidant enzymes can be inhibited by FFAs (Schneider, 1992; Tsoutsikos et al., 2004; Schneider et al., 1993; Mitra et al., 1992; Singh et al., 1988), we speculate that in obesity where high levels of FFA occur, detoxification is further compromised (Barbier et al., 2009; Baijal et al., 1998; Hayes and Mantle, 1986). This presents an empirically sensible pathway to study the etiology of fatty liver disease at the molecular and biochemical level.

Body mass-index (BMI) is a calculated measurement based on height and weight to estimate healthy body weight, assuming average body composition (Equation 1.1). Statistically, a high BMI increases the chances of having pediatric NAFLD.

\[
\text{BMI (kg} / \text{m}^2) = \frac{Weight \text{ in kg}}{Height \text{ in m}^2}
\]

*Equation 1.1. The formula for deriving BMI.*
It is hypothesized that increased visceral fat delivers more FFA directly into the portal circulation, which could lead to increased hepatic fat deposits. Furthermore, visceral fat is also associated with insulin resistance, which can increase de novo lipogenesis. The combination of insulin resistance and visceral fat causes increased levels of hepatic triglycerides and FFAs, leading to net accumulation in hepatocytes (Kawasaki et al., 1997). Despite this, obesity and insulin resistance by themselves do not adequately explain why only certain pediatric populations develop NAFLD. It has been assumed, and often stated; that a genetic component may be involved in predisposing certain populations to NAFLD (Barshop et al., 2009). For example, children from Hispanic, Asian and Native American backgrounds seem to be more disposed to NAFLD (Schwimmer et al., 2006). However, this concept is somewhat confounded by the fact that African-American children, who tend to have higher risk factors for obesity and insulin resistance, have lower prevalence rates for fatty liver disease (Louthan et al., 2005). There also seems to be a familial basis for NAFLD, which is observed at higher rates in families where another member has been diagnosed with NAFLD (Schwimmer et al., 2009). A candidate “genetic basis” for causing NAFLD and progression to NASH may be genetic polymorphisms in detoxification enzymes that confer lower activity. When coupled with direct inhibitory effects of fat, this could provide the impetus for fatty liver disease.
Figure 1.1. Stages of NAFLD through the two-hit hypotheses.
A number of conditions are illustrated to support the first and second hit. Abbreviations: PPARs = peroxisomal proliferation activator receptors, ROS = reactive oxygen species, TNF = tumor necrosis factor, IL = interleukin, TG = triglycerides, FFA = free fatty acids. Modified from Papandreou (2007), with permission from Elsevier.
DIFFERENCES IN PREVALENCE AND HISTOLOGICAL ASPECTS OF PEDIATRIC NAFLD

Although experts assume the pathogenesis of the pediatric and adult forms are similar, the epidemiology of pediatric NAFLD is different from adults. The prevalence is uncertain due to two factors. First, the method for an accurate diagnosis requires a highly invasive procedure. Less invasive procedures are not sensitive and/or not practical in population-based studies. Secondly, selecting a population for study with minimal bias proves difficult. (Patton et al., 2006) For example, based on the National Health and Nutrition Examination Survey (NHANES) that used alanine transaminase as an indicator for NAFLD, it was estimated that 3% of US adolescents have NAFLD compared with 20% of all adults (Lerret and Skelton, 2008). Another study conducting a retrospective review of approximately 800 children at autopsy concluded a prevalence of 10% for NAFLD in children aged 2-19, with 38% of the obese children having fatty liver (Schwimmer et al., 2006). This study may have been over-predictive because other research indicates that only 10% of obese children have NAFLD (Barshop et al., 2009). However, it is difficult at times to compare studies since NAFLD, as a product of obesity has a strong environmental component and comparisons between studies for uncontrolled populations may be confounded by different environmental conditions.

Regardless of their prevalence, since NAFLD is strongly associated with insulin resistance and metabolic syndrome, it is understood that as pediatric obesity rates rise so will the number of cases (Lavine and Schwimmer, 2004). Furthermore, since we are currently in a world-wide obesity epidemic (Hussain and Bloom, 2011), the problem of
pediatric NAFLD and its more extreme progressions will only increase and deserves urgent attention.

Histologically, two forms of pediatric NAFLD have been described: Type I and Type II. Type I, which also manifests in adults, shows a combination of lobular inflammation, ballooning degeneration and perisinusoidal fibrosis with polymorphonuclear leukocyte infiltration (Xanthakos et al., 2006). In contrast, Type II, which is the predominant form observed in children, is characterized by portal inflammation and fibrosis with periportal mononuclear leukocyte infiltration in the absence of ballooning degeneration and perisinusoidal fibrosis (Loomba et al., 2009). One of the most interesting, and perhaps, illuminating points in the difference between children and adults is the location of fibrosis in the liver. In pediatric NAFLD, it seems that fibrosis occurs where the blood enters the liver from the hepatic portal vein. This seems to indicate that toxic products from the gut are damaging the liver, such as bile acids. Bile acids, which are secreted into the intestines, are important for human digestion since they aid in the absorption of dietary cholesterol. However, the detergent properties of bile that aid in cholesterol and fat absorption are also cytotoxic. Hence, there is a tight physiological regulation of bile acids in the human body, with most bile being reabsorbed in the intestines for re-use. It has also been demonstrated that intestinal flora can metabolically convert less toxic bile acids to cytotoxic forms (Baijal et al., 1998).

Of direct importance to this dissertation, UGTs, CYPs and SULTs are vitally important in the regulation of bile acids as they rapidly allow re-secretion of bile acids from the liver.
into the gallbladder via the hepatic duct. Inhibition of these enzymes would lead to
greater levels of bile in the liver, increasing ROS and oxidative stress (Perez and Briz,
2009). Enzymes are not solely responsible for this process since there is a dynamic
interplay between bile detoxification enzymes and canalicular transporters. The important
transporters for bile acid are Na\(^+\) taurocholate cotransporting polypeptide (NTCP), the
bile salt export pump (BSEP), the apical sodium-dependent bile acid transporter (ASBT),
and the organic solute transporter OST\(\alpha\)-OST\(\beta\) (Dawson et al., 2009). Based on the
knowledge that detoxification enzymes can be inhibited by fat, it is empirically sensible
that increased obesity and intracellular fat could start the progression of NAFLD.
Furthermore, because bile acids are being reabsorbed into the blood from the gut, the area
where the bile acids first enter the liver (the portal vein) would likely show the most
damage. This speculation fits the known pathological hallmarks of Type II NAFLD
where there is portal inflammation and fibrosis. Although the clinical implications and
differentiation between Type I and II NAFLD are unknown, histological differences
between the two could indicate that pediatric NASH/NAFLD is a distinct entity with a
unique mechanism.

**PEROXISOME PROLIFERATION-ACTIVATED RECEPTORS AND OBESITY**

Despite our lack of understanding of the precise pathogenesis of NAFLD, there is an
overwhelming clinical association with insulin resistance and obesity. In both of these
cases, FFAs accumulate in the liver, and act as ligands for peroxisome proliferation-
activated receptors (PPARs), a major class of nuclear receptors. While PPARs are not the
only FFA receptors, they are involved in the regulation of intracellular mechanisms
coordinating energy storage and use (George and Liddle, 2008). The PPARs have three isoforms ($\alpha$, $\gamma$ and $\delta$) and respond to environmental cues by binding to free fatty acids and fatty acid derivatives. Receptor-ligand complexes form in the cytosol then translocate to the nucleus where they bind to PPAR responsive elements, coordinating transcription of genes involved in uptake or release of FFA and ultimately modulating lipogenesis and fatty acid catabolism (Andrews et al., 2008).

The PPAR$\alpha$ subtype is involved in lipid metabolism by upregulating fatty acid oxidation and lipolysis and simultaneously decreasing lipogenesis and triglyceride secretion (Yoon, 2009). Regulation of lipid accumulation in the liver by promoting fatty acid uptake and increased fatty acid synthesis is mediated by PPAR$\gamma$, which also increases insulin sensitivity and mediates adipocyte differentiation (Kallwitz et al., 2008). Until recently, PPAR$\delta$ was a mystery. However, recent studies indicate that PPAR$\delta$ is involved in enhancing fatty acid catabolism and energy uncoupling (Luquet et al., 2004). Furthermore, in rhesus monkeys PPAR$\delta$ is activated by polyunsaturated fatty acid ligands, enhancing fatty acid oxidation and adaptive thermogenesis and increasing high-density lipoproteins while reducing inflammatory responses (Barish et al., 2006). It seems that PPAR$\delta$ is activated under oxidative stress conditions, which in turn up-regulate detoxification genes (Coleman et al., 2007). Together, the PPARs play an important role in regulating lipid accumulation, and since this is the first initiation step on the road to NAFLD, PPARs may be important in the molecular mechanisms in the progression of the disease.
PROTECTIVE ROLE OF ANTIOXIDANTS AND DRUG METABOLIZING ENZYMES IN THE LIVER

Antioxidants are compounds that prevent the oxidation of other molecules, such as DNA, proteins and lipids. Oxidation of these molecules damage cellular structures, and can ultimately lead to cell death. This oxidation is caused by ROS, oxygen containing free radicals that highly react with molecules other than molecular oxygen. Under normal physiological conditions, ROS are constantly being produced in the body and can also be initiated by pollutants coming from outside the body. Originally, ROS were only thought of as a toxic by-product of aerobic respiration or pollution (Sorg, 2004). Subsequently, it has been recognized that the roles of ROS are far more complex. For instance, ROS are used in cell signaling through the NF-κB pathway, are involved in nitric oxide-mediated vasodilation and for innate host defenses in neutrophils and other immune cells (Hensley et al., 2000). Examples of ROS include superoxide, hydrogen peroxide, nitric oxide, hypochlorite, and metal ions (Cu$^{2+}$ and Fe$^{3+}$) (Sorg, 2004).

These species are usually scavenged by a suite of antioxidant enzymes. This network encompasses as superoxide dismutate (SOD), catalase (CAT), thioredoxin reductase (TR), NADPH-dehydrogenase oxido-reductase quinine 1 (NQO1), glutathione peroxidase (GPx), glutathione reductase (GR), and GST. Non-enzymatic antioxidant scavengers can bind and sequester them, including vitamin E, vitamin C, glutathione (GSH/GSSH), and bilirubin (Figure 1.2). However, when the generation of ROS exceeds the body’s ability to neutralize and eliminate them, damage to DNA, proteins, carbohydrates, and lipids occurs; this is known as oxidative stress (Tsukahara, 2007).
There are other enzymes, such as CYPs, UGTs and SULTs, that also indirectly reduce ROS. The CYP, UGT and SULT families have low-affinity to many substrates; this means the enzymes are able to metabolize many different chemicals, both endogenous (e.g. steroids and hormones) and exogenous (e.g. drugs and pesticides), with high efficiency. By removing compounds that can directly create ROS, all of these “metabolizing” enzymes can also help to protect the liver. For example, the isoforms UGT1A3, 1A4, 2B4, and 2B7 are directly involved in clearing bile acids from the body. Bile acids, if not excreted, create ROS and subsequently damage the liver.

Since FFAs inhibit UGTs, this may allow the buildup of bile acids and ROS. Excessive ROS impairs hepatic detoxification, allowing buildup of more toxic products (i.e. bile acids) and initiate a futile cycle that leads to liver disease (Barbier et al., 2009). This is most well demonstrated, for example in primary biliary cirrhosis, where the bile duct is physically blocked and does not allow the excretion of bile into the intestines and results in eventual scarring, fibrosis and cirrhosis (Bezerra, 2006). When these facts are considered together, it is likely that increased oxidative stress occurs when UGT enzymes are inhibited, either collectively or for individual isoforms.

Despite the importance and magnitude of hepatic detoxification, the developmental dynamics of these enzymes, particularly the UGTs, are not well-studied. Neither has the ontogeny of antioxidants and antioxidant enzymes been well elucidated even though they play a pivotal role in overall human development. Ontogenetic studies are especially important from a regulatory standpoint. Medications are already prescribed that have not
been adequately studied for safety in pediatric medicine, and as diseases of childhood continue to move from acute to chronic etiologies, the number of pediatric adverse drug reactions will only increase. Regulatory bodies are increasingly requested to raise pediatric exposure limits and safety levels for environmental chemicals, commonly without good human data. Studies such as those described here can contribute by describing pediatric development and also by providing a relevant framework for the understanding of developmental physiology and pharmacology.
Figure 1.2. Overview of the antioxidant network.
Free radical/ROS formation starts with molecular oxygen to superoxide or to the semiquinone forms of ubiquinol. The hydrogen peroxide is produced that gives rise to hydroxyl radicals, which can initiate lipid peroxidation. Abbreviations: 6PGδL, 6-phosphoglucono-δ-lactone; A, an oxidized substrate; AH₂, a reduced substrate; Arg, arginine; ASC, ascorbate; ASC•, semi-dehydroascorbate; Cat, catalase; Cit, citrulline; DHA, dehydroascorbate; G6P, glucose 6-phosphate; GPX, glutathione peroxidase; GSH, reduced glutathione; GSSG, oxidized glutathione; GSR, glutathione reductase; GST, glutathione-S-transferase; HK, hexokinase; LH, a lipid, L•, a lipid radical; LOH, an hydroxylated lipid; LOO•, a lipid peroxyradical; LOOH, a lipid hydroperoxide; LSG, a glutathione conjugated lipid; NOS, nitric oxide synthase; NO•, nitric oxide; NQO1, NADPH-dehydrogenase oxido-reductase quinine 1; PS, photosensitizer; Q, ubiquinone; QH•, ubisemiquinone; QH₂, ubiquinol; TOC, α-tocophorol, TOC•, α-tocopheryl radical; Trx, thioredoxin reductase. Modified from Sorg (2004), with permission from Elsevier.
SUMMARY

While it is assumed that the pathogenesis for pediatric and adult NAFLD are the same, concrete evidence is lacking. Furthermore, although the “two-hit” hypothesis seems to be reasonably well accepted, it remains a theory and has not been definitively proven. Specific and focused research efforts into NAFLD causes and promoters will be essential to elucidate the broad trends in fatty liver diseases as well as nuances in the molecular mechanisms of this disease. This is particularly important for children since the pediatric form of NAFLD seems to be epidemiologically distinct. Separate consideration of genetic and environmental influences will be a key factor in helping to determine risk factors for this disease. By understanding the enzymatic profiles hepatic detoxification enzymes in the pediatric liver, we can begin to design strategies to prevent and/or reverse pediatric NAFLD. This will increase the quality of life for both obese children and adults, including preventing further progression to life-threatening conditions.

In conclusion, the ontogenies of many detoxification enzymes, particularly the UGTs, are poorly understood. The goals of this dissertation are to characterize the ontogeny of antioxidant and bile-acid detoxification enzymes. By understanding how and when these enzymes develop, the molecular mechanisms of pediatric NAFLD can be fully investigated. Data gained from these studies can be used to modify current drug administration and dosages to reduce risks associated with adverse drug reactions and toxicities in children. Furthermore, by investigating the interplay between pediatric antioxidant capacity, the maturation of metabolizing enzymes and obesity-mediated inhibition of detoxification, we may elucidate pivotal interactions that cause or contribute
to pediatric NAFLD and formulate strategies (pharmacological or otherwise) for preventing and treating this disease.
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CHAPTER 2

DEVELOPMENTAL CHANGES IN HEPATIC ANTIOXIDANT CAPACITY ARE AGE- AND SEX-DEPENDENT

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ABSTRACT

Developmental inadequacy in hepatic antioxidant defenses may contribute to chemical toxicity and pediatric liver diseases. We measured a comprehensive panel of antioxidants in liver tissue from 27 normal pediatric donors. Glutathione reductase declined with age (P = 0.008, r = -0.54, Spearman) while microsomal glutathione-S-transferase increased (GST, P < 0.001, r = 0.81). Males had significantly lower superoxide dismutase and Vitamin E (P < 0.05) and may have lower glutathione reductase (P = 0.06), while females show less cytosolic GST (P = 0.07). Hepatic antioxidants are high in neonates, decline throughout childhood then increase in adolescence to adult levels.
Reactive species, including reactive oxygen species and chemical metabolites, are generally prevented from causing cell and organ damage through the actions of a coordinated set of antioxidant enzymes and compounds. This “antioxidant defense network” prevents hepatocellular damage and ultimately protects against many systemic toxicities (Aboutwerat et al., 2003; Tsukahara, 2007).

Since the development of pediatric liver detoxification is largely unknown, we measured a comprehensive panel of antioxidant enzymes and compounds in normal liver tissue from 27 pediatric donors aged 13 days – 20 years. These samples were derived from post-mortem donors with healthy livers (Xenotech, Lenexa, KS) and their use was approved by the University of Hawaii Institutional Review Board for Human Subjects. Because tissues were collected post mortem and ischemia causes high levels of oxidative stress, we avoided total antioxidant status or reactive species assays. However, ischemia has lesser effects on antioxidant enzymes and compounds. For example, hepatocytes can be extracted from liver up to 8 hrs post-mortem yet still possess enzyme activities that have a high degree of concordance with in vivo activities (Guillouzo et al., 1993).

In this study, we investigated glutathione-dependent antioxidant enzymes: Glutathione Peroxidase (GPx), Gluthathione Reductase (GR, commercial kits from Cayman Chemical Company, Ann Arbor MI) and Glutathione-S-transferase (GST, cytosolic and microsomal forms), assayed as previously described with the substrate 1-chloro-2,4-dinitrobenzene (Habig et al., 1974). Additionally, non-glutathione antioxidant enzymes Catalase (CAT), Superoxide Dismutase (SOD) and Thioredoxin Reductase (TR) were
analyzed using Cayman Kits and Quinone Oxido-reductase (NQO1) was measured with dichloroindophenol and dicumarol as previously described (Ernster et al., 1972).

Quality control samples were prepared from pooled adult human livers (n = 50, Xenotech, Lenexa, KS) assessed in triplicate to gauge the precision of each method and also provide a comparison to average antioxidant activities in adults. This pooled sample comprised of Hispanic (8%), African American (2%) and Caucasian (90%) donors, 25 each men and women with an average age of 48.5 yrs (ranging: 21-69). The coefficients of variation (CV) for each assay were: CAT: 9.5%, GPx: 10.7%, GR: 7.5%, SOD: 9.0%, and TR 15.7% for kit assays. For biochemical assays, intra- and inter-day CV were 2.6% and 3.8% for cytosolic GST, 5.3% and 7.9% for microsomal GST and 10.6 and 11.2% for NQO1.

The activities of several hepatic glutathione enzymes differed with age and sex in pediatric liver samples. Although linear correlations with age were not significant for GPx and cytosolic GST, a consistent pattern showing highest activities in neonates and adults with lower activities between 1 and 10 years was observed (Figures 2.1a and 2.1e). The activity of GR was significantly negatively correlated with age, being highest under one year of age (P = 0.0077, r = -0.54 Spearman, Fig 2.1c). The most convincing and startling findings of this study were results from microsomal GST activities in pediatric liver. Microsomal GST activities were extremely low in neonatal liver and increased significantly as children aged, appearing to plateau some time in the teenage years (P < 0.0001, r = 0.81 Spearman, Figure 2.1g). Males had consistently lower GR (P = 0.06,
Figure 2.1d) and microsomal GST (P = 0.10, Figure 2.1h) activities than females, which may become significant with a larger sample size. The opposite is true for cytosolic GST, where females had consistently less GST activity than males (P = 0.07, Figure 2.1f).

For non-glutathione antioxidant enzymes, SOD enzymes differed significantly with age and gender. Children under 1 have significantly higher SOD activity than children aged 6-10 but rates of neonatal SOD activity are not different than hepatic SOD activities in those aged 11 and older (Figure 2.2a, Bonferroni’s multiple comparison test). SOD was also significantly lower in males than females (P < 0.05, Figure 2.2b). CAT, NQO1 and TR did not change with age or gender (Figure 2.2c – h).

There are few clinical investigations of hepatic antioxidant status in children and even fewer comparing antioxidant status to liver disease in humans. In one study, significantly lower SOD activity was reported in the blood of children with Wilson’s disease and levels of SOD activity were inversely proportional to disease severity (Nagasaka et al., 2006). Additionally, children with cystic fibrosis who also have decreased activity in the GSTP isoform were eight-fold more likely to develop biliary disease (Henrion-Caude et al., 2002). Finally, adult patients with non-alcoholic fatty liver disease have significantly lower hepatic glutathione content, as well as significantly lower SOD and CAT activity compared to normal liver tissue (Debier, 2007). Hence, these disease-specific studies strengthen our speculation that developmentally compromised antioxidant status may be related to the etiology of pediatric liver diseases.
Figure 2.1. Changes in glutathione-related antioxidant enzymes are age- and gender-related in pediatric liver tissue.
A: The activity of GPx with age. B: The activity of GPx with sex. C: GR enzyme activity with age. D: GR enzyme activity with sex. E: cGST activity with age. F: cGST activity with sex. G: mGST enzymes with age. H: mGST activities with sex. Categories for cytosolic enzymes correspond to n = 8 (0-1), n = 4 (2-5), n = 6 (6-10) and n = 5 (11-20) and for microsomal enzymes n = 8 (0-1), n = 4 (2-5), n = 9 (6-10) and n = 6 (11-20). a = P < 0.05 vs. 0-1 using Bonferroni’s multiple comparison test. Bars are means ± SD of triplicate determinations for each liver. * = P < 0.05, male vs. female, students t-test. Dashed line = 95% confidence interval.
Figure 2.2. The activities of SOD but not other non-glutathione antioxidant enzymes in the cytosol are significantly age- and gender-related in pediatric liver tissue. A: Changes in SOD activity with age. B: SOD activities with sex. C: CAT activity with age. D: CAT activity with sex. E: NQO1 with age. F: NQO1 with sex. G: TR with age. H: TR with sex. a = \( P < 0.05 \) vs. 0-1 using Bonferroni’s multiple comparison test. * = \( P < 0.05 \), student’s t-tests. N values for each category are as listed in Figure 2.1.
In addition to antioxidant enzymes, reactive species can also be removed through binding and sequestration by antioxidant compounds such as glutathione, Vitamins C and E (Tsukahara, 2007). In neonates, the accumulation of antioxidant compounds is highly complex and begins before birth. For example, the fat-soluble nutrient and an antioxidant compound Vitamin E is mainly present in the neonate through placental active transport that occurs in third trimester of pregnancy (Debier, 2007). In contrast, Vitamin E is primarily accumulated in children through diet (Debier, 2007). Pre-term infants can have very low Vitamin E status and high levels of oxidative stress which are both implicated in sepsis, respiratory distress and retinopathy (Tsukahara, 2007; Debier, 2007). Therefore, appropriate systemic levels of neonatal and pediatric antioxidant compounds are very important.

Because blood constantly circulates and may gain or lose compounds rapidly via distribution, metabolism and elimination, measuring antioxidant vitamins and minerals directly in liver tissue may present a more stable and accurate picture of hepatic detoxification capacity. Therefore, we measured total glutathione (Cayman Chemical Company Kit, Ann Arbor MI) as well as Vitamin E (Tütem et al., 1997) and Vitamin C (Omaye et al., 1979) in liver S9, which is a sub-cellular fraction that contains both microsomes and cytosol but not nuclei or mitochondria. The precision (quality control CVs) for these assays were 0.2% glutathione, 4.8% Vitamin E and 0.8% for Vitamin C. Our results demonstrated that the levels of hepatic glutathione, Vitamins C and E were not significantly affected by age (Figures 2.3a – c). Vitamin E was significantly lower in males than females (P = 0.015, t-test, Figure 2.3b) but glutathione and Vitamin C were
not (Figures 2.3a and 2.3d).

Figure 2.3. Differences in antioxidant compound levels are not age-dependent but some gender differences occur in pediatric liver tissue.
A Hepatic vitamin C levels with age. B: Hepatic vitamin C levels with sex. C: Hepatic vitamin E activities with age. D: Hepatic vitamin E activities with sex. E: Total hepatic glutathione content with age. F: Total Hepatic glutathione with sex. Bars are means ± SD and n values for each category are as listed in Figure 1. * = P < 0.05, t-test. N values for each category are as listed in Figure 2.1.
The most striking findings of this study are the age-related changes occurring in antioxidant enzymes in the pediatric liver. All of the cytosolic antioxidant enzymes, both glutathione and non-glutathione related, showed high activity in the neonatal liver that declines during childhood with a nadir around 6-10 years and then increased to adulthood. For GPx and SOD, significant decreases were apparent and for the remaining enzymes a consistent trend was observed. In contrast, the microsomal GST enzymes that are anchored in the endoplasmic reticulum, show sparingly low activity in neonatal liver that increases with age, apparently peaking to adult levels in adolescence. Microsomal GST enzymes have a distinct physiological role in addition to detoxification: they are integrally involved in eicosanoid and leukotriene signaling and the production of pro-inflammatory stimuli (Jakobsson et al., 1999; Hayes et al., 2005; Dwivedi et al., 2007). Since the inflammatory and immune systems are not fully developed until late childhood, it follows that inflammatory recruitment enzymes such as microsomal GST develop to the same time line.

Although small sample size is a limitation of this study, the study’s strength is that the tissues derive from normal livers. Most large studies present data from patients undergoing surgery for abnormalities and these studies cannot rule out confounding of their results secondary to disease (Strassburg et al., 2002). Thus, these data represent a valuable contribution to defining developmental dynamics in the normal pediatric population. Furthermore, the high level of significance despite the small sample size, argues strongly for the robustness of the data. Oxidative stress is an initiating event for liver injury. Mechanistically, bile acids and fat
accumulate in hepatocytes leading to pathological changes in liver architecture and function that cause clinical symptoms. Our results showing declining antioxidant capacity in childhood that is more pronounced in males, are compatible with this mechanism and also with the clinical incidence of pediatric fatty liver disease which is mainly diagnosed in males between the ages of 11 and 14 (Bezerra, 2006). The pro-inflammatory role of microsomal GST enzymes also fits this paradigm because when hepatic detoxification capacity is least effective, microsomal GST peaks, increasing inflammatory stimuli and the potential for hepatocyte damage (Hayes et al., 2005; Dwivedi et al., 2007).

The incidence of liver disease in children is increasing and fatty liver disease is becoming one of the most serious clinical concerns in childhood (Bezerra, 2006). This study demonstrates that antioxidant capacity in children differs with age and gender and while neonates may be well protected, children under ten have particularly low hepatic detoxification ability in comparison to either neonates or adults. We believe this provides insight into potential mechanisms for altered pediatric response to drugs and other xenobiotics and for the developmental basis of pediatric liver disease. Additional studies should focus on investigating differences in detoxification enzymes between normal and diseased liver, with and without obesity, to confirm and expand upon these findings. Furthermore, research into nuclear receptors and signaling pathways activated by hepatic oxidative stress and fat accumulation, will be useful to define the mechanisms behind these changes and provide targets for pharmacological and clinical intervention.
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CHAPTER 3

PEDIATRIC DEVELOPMENT OF GLUCURONIDATION:
THE ONTOGENY OF HEPATIC UGT1A4

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ABSTRACT

This article reports on the development of UDP-glucuronosyltransferase (UGT) enzyme activity in pediatric livers. The substrates 4-methylumbelliferone (4MU) and trifluoperazine (TFP) were used as probes for general glucuronidation and specific UGT1A4 activity respectively. The activity of hepatic \( \beta \)-glucuronidase enzymes was also determined so as to investigate the balance between glucuronide clearance and systemic re-circulation. UGT activity towards 4MU reached maximum levels by 20 months of age, while the activity of \( \beta \)-glucuronidase was highest in the neonatal liver and decreased to steady-state adult levels by 4 months. The average \( V_{\text{max}} \) and \( K_m \) for UGT1A4 in pediatric samples were 151.9 ± 63.5 pmol/min/mg protein and 14.4 ± 9.6 µM respectively. Average \( V_{\text{max}} \) was understandably low due to developmental dynamics, but \( K_m \) was similar to those reported elsewhere. When a constant rate of enzyme development is assumed, maximum activity of UGT1A4 occurs at 1.4 years of age. When the intrinsic hepatic clearance of TFP was scaled with an allometric model, hepatic clearance of TFP by UGT1A4 did not reach maximum levels until 18.9 years of age and scaled results underestimated reported \textit{in vivo} clearances in adult males. No significant differences in UGT activities or hepatic clearances were observed with gender or ethnicity. The developmental dynamics of most drug metabolizing enzymes are unknown and this paper contains the first description of the development of a single UGT isoform in childhood. Ultimately work such as this is important for predicting drug responses and for developing and evaluating new medications in children.
INTRODUCTION

One of the main causes of adverse drug reactions in children is believed to be a lack of substantial drug metabolism due to immaturity of drug metabolizing enzymes (Benedetti et al., 2005). Although the vast majority of drug metabolism is performed by enzymes in the human liver, developmental profiles for most drug metabolizing systems in the liver (and other organs) are not defined.

Within the many families of metabolizing enzymes, the UDP-glucuronosyltransferase (UGT) super family is critical for the metabolic clearance of most biological substances including drugs, dietary, environmental and endogenous compounds (Radominska-Pandya et al., 1999; Williams et al., 2004). In the liver, the UGT super family is divided into the UGT1A and UGT2B subfamilies that contain nine and seven isoforms respectively (Radominska-Pandya et al., 1999; Levesque et al., 2001). They are high capacity, low affinity enzymes that demonstrate considerable overlapping affinity for multiple substrates; however, some substrate specificities do occur. Of the known UGT isoforms, twelve are expressed in the adult human liver and three have no known physiological or xenobiotic substrates (Radominska-Pandya et al., 1999; Levesque et al., 2001). Thus, biologically relevant drug and hormone metabolism is mediated by the remaining nine isoforms.

Within the UGT1A subfamily, UGT1A4 is one of the lesser-known isoforms. It was first cloned by Ritter et al. in 1991, shows high sequence homology with UGT1A3 and is expressed in the gastrointestinal system (Ritter et al., 1991; Radominska-Pandya et al.,
The isoform is active towards primary, secondary, and tertiary amines, aromatic amines, androgens, progestins and plant steroids (Mori et al., 2005). In terms of clinically used drugs, UGT1A4 metabolizes the antidepressants amitryptiline and imipramine, the antipsychotic clozapine, quinine antimalarials and tamoxifen (Radominska-Pandya et al., 1999; Ogura et al., 2006; Uchaipichat et al., 2006a).

Despite playing a role in placental dynamics during gestation, UGTs are largely absent from the fetal liver (Coughtrie et al., 1988; Collier et al., 2002a; Collier et al., 2002b). Their subsequently low activity in the neonate and the child’s inability to excrete bilirubin is the major cause of jaundice in newborns (Onishi et al., 1979; Kawade and Onishi, 1981; Strassburg et al., 2002). Despite this serious effect, the developmental dynamics of UGTs in neonates have not been defined. Kawade and Onishi showed that premature and term neonates develop UGT activity at the same rate from the neonatal period up to 6 months of age, implying a level of environmental as well as genetic control (Onishi et al., 1979; Kawade and Onishi, 1981). More recently, Strassburg et al. have demonstrated that while mRNAs for all hepatic UGTs are expressed within 6 months, even at the age of two, UGT activities are up to 40-fold lower than adults’ (Strassburg et al., 2002). After the age of 2, further development of UGT enzymes in children has not been previously reported.

Although it is well recognized that children have altered drug disposition, a comprehensive picture of their pharmacokinetics is lacking (Benedetti et al., 2005). Because children are commonly switched from pediatric to adult dosing schedules at or
around 12-14 years old without noticeable adverse effect, it has been assumed that drug metabolism likely reaches full adult activity sometime during early childhood. However, recent reports, such as those highlighting adverse reactions of teenagers and adolescents to anti-depressant drugs, seem to contradict this assumption (Duff, 2003).

Thus, if the nominal age of adulthood is at or around 20 years, we supposed that UGT enzymes, being a major path of drug metabolism, must develop before this time. To test our hypothesis, we determined total UGT activity in sub-cellular fractions (microsomes) from 27 normal pediatric liver samples (0 – 20 years of age) and compared these to UGT activity in pooled adult liver fractions. We used one compound, 4-methylumbelliferone (4MU); that is metabolized by all the hepatic UGT isoforms except UGT1A4 (Uchaipichat et al., 2004) and another, the antipsychotic drug trifluoperazine (TFP), that is a specific substrate for UGT1A4 alone (Uchaipichat et al., 2006a). We also determined the activity of hepatic β-glucuronidase enzymes to assess the relative balance between conjugation/excretion and hydrolysis/enterohepatic re-circulation of drugs in children and neonates.

MATERIALS AND METHODS

Materials

4-methylumbelliferone sodium salt (4MU), 4-methylumbelliferone glucuronide (4MUG), alamethicin (from trichoderma viridae), β-glucuronidase (from E. coli), MgCl2, D-saccharic acid 1,4-lactone (saccharolactone), trifluoperazine hydrochloride (10-[3-(4-Methylpiperazin-1-yl)propyl]-2-(trifluoromethyl)-10H-phenothiazine dihydrochloride,
TFP), Tris-HCl and uridine diphosphoglucuronic acid (UDPGA) were purchased from Sigma Chemical Company (St Louis, MO). Hecogenin was from Sciencelab.com Inc. (Houston, TX).

**Pediatric and Adult Liver Samples and Demographics**

Pediatric and adult liver microsomes were purchased from Xenotech LLC (Lenexa, KS) and were derived from post-mortem donors with healthy livers. Pediatric microsomes (27) were derived from single livers and our study included Asian (4%), Hispanic (11%), African American (19%) and Caucasian (67%) descent, with 10 female and 17 male donors. Pooled adult liver microsomes were derived from 50 donors and contained Asian (4%), Hispanic (6%), African American (6%) and Caucasian (84%) ethnicities with equal numbers of men and women (25 each). The average age was 50 with a range of 17-78.

**Total UGT activity with 4MU**

The assay for UGT activity with 4MU was carried out as previously described (Collier et al., 2000). Three reactions were performed per sample and samples were assessed on 3 separate days. Initial reaction velocities were calculated by least squares linear regression with substrate depletion as a marker of product formation. Fluorescence units (FU) were converted to concentrations by comparison to a standard curve of 4MU. The average $r^2$ and slopes of the standard curves were $0.98 \pm 0.01$ and $550.5 \pm 12.9$ FU/µM respectively ($n = 6$). The intra- and inter assay CVs for the slopes of the standard curves were 3 and 8 % respectively and for pooled adult human liver microsomes (positive control) 14.8 and 16.1% respectively.
De-conjugation (β-glucuronidase) activity

The assay for β-glucuronidase activity was performed with 4-methylumbelliferone glucuronide essentially according to the method of Trubetskoy and Shaw (Trubetskoy and Shaw, 1999). Briefly, microsomes (0.1 mg) and Tris-HCl containing 5 mM MgCl₂ were placed in a microplate and pre-warmed to 37 °C for 2 minutes. The reaction was started by addition of 4-methylumbelliferone glucuronide (4MUG) to a final concentration of 100 µM. Reactions were monitored continuously at 37 °C for 20 minutes at λ = 355 nm excitation and λ = 460 nm emission (Gemini XS, Molecular Devices, Sunnyvale CA). Three reactions were performed per sample on 3 separate days. Initial reaction velocities were calculated by least squares linear regression using the appearance of fluorescence. FU were converted to concentrations by comparison to a standard curve of 4MU. Each day a positive control (0.2 mg β-glucuronidase from *E. coli*), negative control (containing 5 mM sacchrolactone) and reference comparison (pooled adult human liver microsomes of n = 50 donors) were performed. Negative controls averaged 0.07 ± 0.3 pmol/min/mg activity – essentially zero. Positive controls averaged 0.5 ± 0.24 pmol/min/mg protein and pooled adult human liver microsomes 1.7 ± 0.9 pmol/min/mg protein. The accuracy and precision of the standard curves was the same as reported above for 4MU activity.

Measurement of UGT1A4 activity

UGT 1A4 activity was measured using the assay conditions described by Uchaipichat et al. (2006b) with detection of glucuronidation performed by monitoring the fall in fluorescence in solution (substrate depeletion) at wavelengths described by Rele et al.
Briefly, reactions were carried out in 0.1 ml containing 0.2 mg/ml microsomal protein, 2 mM UDPGA, 10 mM MgCl₂, 0.025 mg/ml alamethicin and 0-200 µM TFP in 50 mm Tris (pH 7.5). Reactions were incubated at 37 °C for 20 minutes in the dark and detection of activity was performed fluorimetrically in a Gemini XS microplate spectrophotometer (Molecular Devices, Sunnyvale, CA) at λ = 310 nm excitation and λ = 475 nm emission (Rele et al., 2004). Each sample was assessed in triplicate. Substrate depletion was used to measure UGT1A4 activity as fluorescence became masked by glucuronidation. Quantitation was achieved by comparison to standard curves that were prepared fresh each day in shielded tubes. The average r² for standard curves was 0.985 ± 0.010 with intra- and inter-day CV of 3.5 and 3.6% respectively (n = 6, in triplicate).

To confirm that the loss of fluorescence was specifically due to glucuronidation of TFP, we performed triplicate incubations as described above except that incubations proceeded for 1 hour at 37 °C. One set of incubations contained microsomes and 100 µM TFP with no UDPGA and the second contained microsomes and 100 µM TFP with UDPGA. A third incubation proceeded for 1 hr after which we added 1000 Units of β-glucuronidase enzyme and continued the incubation for 30 minutes. Subsequently the emission spectra (excitation λ = 310 nm) for each set of incubations were scanned from 350 – 500 nm (Gemini XS, Molecular devices, Sunnyvale CA).

The specificity of glucuronidation was confirmed with pooled adult human liver microsomes using the incubation conditions described above with 100 µM TFP substrate and the specific UGT1A4 inhibitor hecogenin (200 µM in ethanol, (Uchaipichat et al., 2006a). Total solvent amounts did not exceed 1% of the individual incubations.
Pharmacokinetics, Scaling and Statistical Analyses

Data for 4MU and 4MUG were performed at a single concentration of substrate (100 µM) and velocities were plotted directly. Experimental data for TFP were derived from 11 concentrations of TFP (and a blank) per sample and were performed at least twice, in triplicate. They were subsequently fit to the Michaelis-Menten equation using Prism 3.0 (GraphPad, San Diego, USA). There has been some discussion of the correct interpretation of UGT1A4 kinetics based on whether the enzyme exhibits substrate inhibition by TFP at higher concentrations (Uchaipichat et al., 2006a). We fit our data to both Michaelis-Menten kinetics and the equation for substrate kinetic inhibition defined by Houston and Kenworthy and compared these with an F test for suitability (Houston and Kenworthy, 2000). Of the 65 fits performed, only 20 (31%) were better fit to the substrate inhibition equation. Subsequently all TFP data were fit to the Michaelis-Menten models

Because concentration-dependent non specific binding of TFP is known to occur under the conditions used, kinetic data were analyzed with respect to the unbound fraction of drug only (f_u) using published data for protein binding (Uchaipichat et al., 2006b).

To evaluate hepatic drug clearance, we scaled our enzyme kinetic results using both the well-stirred and parallel tube models (Equations 3.1 and 3.2).
\[
CL_{hepatic} \ (L/ hr) = \frac{Q_{hepatic} * f_u * CL_{int}}{Q_{hepatic} + f_u * CL_{int}}
\]

Equation 3.1. The well-stirred model.

\[
CL_{hepatic} \ (L/ hr) = Q_{hepatic} * \left(1 - e^{-\frac{-CL_{int} * f_u}{Q_{hepatic}}} \right)
\]

Equation 3.2. The parallel tube model.

Here, \(Q_{hepatic}\) is hepatic blood flow, \(CL_{int}\) is intrinsic clearance and \(f_u\) is the unbound fraction of drug. Intrinsic clearances were generated by using experimental intrinsic clearances \((V_{max}/K_m)\), assuming liver size of 1500 g, a hepatic flow rate of 1.5 L/min, protein content of 45 mg/g and plasma unbound fraction for TFP of 0.05. (Midha et al., 1983; Verbeeck et al., 1983; Houston, 1994). Subsequently an allometric model was needed to scale clearance (Equation 3.3) to children’s weight:

\[
CL_{pediatric} \ (L/ hr) = CL_{hepatic} * \left(\frac{W_i}{W_{std}}\right)^{\frac{3}{4}}
\]

Equation 3.3. Allometric model.

Where \(W_i\) is the weight of the individual and \(W_{std}\) was taken from the adult (20 years) weights of men and women from the same charts. Age and gender were known for each donor; thus, average weights for age and gender were taken from the National Center for Health Statistics growth charts (Statistics., 2000) and substituted for the variable \(W_i\) to
scale hepatic clearance for individual donors.

To define adult levels of enzyme activity in the population, one-phase exponential association (4MU, TFP, Equation 3.4) or one-phase exponential decay (4MUG, Equation 3.5) non-linear least squares regression equations were used. These models assume a constant rate of change (K) in the development of the enzyme/s that fits a curve ending in a plateau rate or action. A table of XY co-ordinates defining the curve was generated and the “age of adult activity” was defined as the youngest age at which plateau levels were reached.

\[ Y = Y_{\text{max}} \times (1 - e^{-KX}) \]

Equation 3.4. One-phase exponential decay.

\[ Y = \text{Span} \times e^{-KX} + \text{plateau} \]

Equation 3.5. One-phase exponential decay.

Goodness of fit was assessed with F tests, r² values, absolute sums of squares and Sy.x.

RESULTS

Balance between Glucuronidation and Hydrolysis in the Pediatric Liver

The balance of metabolism in neonatal life shifts from metabolite cleavage and systemic recirculation in the neonate to detoxification in the child. Assuming a constant rate of increase in UGT activity, the combined activity of UGT isoforms towards 4MU increased
to maximum adult levels of 1.53 nmol/min/mg protein by 20 months of age (Figure 3.1a).
When compared to the activity measured in pooled adult human liver microsomes (2.35 ±
0.38 nmol/min/mg protein), the maximum adult UGT activity from the model showed
reasonable agreement with measured UGT activity. No significant differences between
genders and ethnicities were reported (P = 0.3655, t-test and P = 0.5678 ANOVA
respectively).

The activity of β-glucuronidase was highest in the neonatal liver and decreased to steady-
state adult levels of 1.61 pmol/min/mg protein by 4 months of age (Figure 3.1b). The
measured activity in pooled adult liver microsomes of 1.7 ± 0.9 pmol/min/mg protein
showed excellent agreement with the adult activities derived by the model. No
significant differences between genders or ethnicities were observed for either enzyme (P
= 0.2258 t-test and P = 0.3715, ANOVA respectively).
Figure 3.1. The balance of total glucuronidation and deconjugation in the pediatric liver with age.
A: UGT activity towards the pan-specific substrate 4MU (100 µM). Plateau is reached at 1.7 years (20 months) and apparent mean adult activity is 1.53 nmol/min/mg protein. Bars are means of n = 3, in triplicate ± SD. 1B: β-glucuronidase activity towards 4MUG. Plateau is reached at 4 months of age and adult activity of the enzyme is 1.61 pmol/min/mg protein. Points are means of n = 3, in triplicate ± SD.
Pediatric Development of UGT1A4

After adjustment for non-specific binding to microsomal protein, the derived \( V_{\text{max}} \) and \( K_m \) in pediatric samples averaged 151.9 ± 63.5 pmol/min/mg protein (range 61.6 – 276.8) and 14.4 ± 9.6 µM (range 5.4 – 42.3 µM) respectively. This compares with the kinetic parameters measured from pooled human adult liver microsomes of 348.2 ± 14.93 pmol/min/mg protein and 15.86 ± 1.98 µM for \( V_{\text{max}} \) and \( K_m \) respectively. A typical kinetic plot for one pediatric liver sample is presented in Figure 3.2a.

Hecogenin suppressed UGT1A4 activity in pooled adult human liver microsomes. The activity of TFP alone in pooled human liver microsomes (50 donors) was normalized to 100% and when hecogenin was included in incubations, activity was 31.6 ± 9.0 % of control levels (n = 3, in triplicate Figure 3.2b).

Assuming a constant rate of development, UGT1A4 activity reached maximum (adult) levels of 113.1 ± 10.17 pmol/min/mg protein at 1.4 years of age (Figure 3.3a) This modeled maximum activity was somewhat lower than the measured \( V_{\text{max}} \) of UGT1A4 in pooled adult microsomes of 230.5 ± 15.9 pmol/min/mg protein. TFP glucuronidation did not differ significantly with gender or ethnicity (P = 0.7758, t-test and P = 0.2258, ANOVA respectively).

The well-stirred and parallel tube models returned the same maximum clearances and times to adult activities. When data were scaled to pediatric hepatic clearance using the allometric model, apparent adult clearance was reached at 18.9 years with a modeled
maximum hepatic clearance of $0.357 \pm 0.037$ L/hr (range 0.05 – 1.2 L/hr, Figure 3.3b).
This shows moderate correlation with the adult clearance experimentally gained from liver microsomes of 0.80 L/hr.
Figure 3.2. UGT1A4 activities in pediatric liver.

A: Typical kinetic graph of UGT1A4 in one human liver sample. In this sample, $V_{\text{max}} = 212.7 \pm 12.4$ pmol/min/mg protein, $K_m = 12.28 \pm 1.96 \mu$M. Points are means of triplicate determinations $\pm$ SD. B: Emission spectra for Trifluoperazine with and without microsomal incubations (excitation at 310 nm). Spectra were generated from 1 hr incubations (in triplicate) performed as detailed in materials and methods. The three spectra are derived from incubations containing microsomes and TFP only (solid line) and Microsomes, TFP and UDPGA (dotted line). To prove the loss of fluorescence was due to glucuronide conjugation, after 1 hour 1000 Units of $\beta$-glucuronidase were added to the third incubation and further incubated for 30 minutes (dashed line). Recovery of fluorescence can be observed. C: Inhibition of UGT1A4 activity in pooled adult human liver microsomes by hecogenin (100 $\mu$M). Average activity was $31.6 \pm 9.0$ % of control levels. Bars are means $\pm$ SD of 3 experiments, each performed in triplicate.
Figure 3.3. The development of UGT1A4 activity in the pediatric liver.  
A: The development of UGT1A4 activity in the pediatric liver ($V_{\text{max}}$). Apparent maximum adult activities are reached at 1.4 years with apparent mean adult activity at $113.1 \pm 10.17$ pmol/min/mg protein (range $62.7 – 268.9$ pmol/min/mg protein). The model for UGT1A4 development is weighted by $1/Y^2$ and constrained by strict convergence criteria requiring five consecutive iterations of the fit to change the sum-of-squares by less than 0.000001%.  
B: The scaled hepatic clearance of TFP in neonates and children aged 13 days to 20 years using an allometric model. Plateau is reached at 18.9 years with an apparent mean adult clearance of $0.357$ L/hr (range $0.05 – 1.3$ L/hr). The model is unweighted and standard criteria for convergence are applied.
DISCUSSION

The balance of glucuronide metabolism shifts very early in the neonate from cleavage and re-circulation to conjugation and clearance. The activity of β-glucuronidase is initially high, but decreases from birth to reach apparent adult levels by 4 months of age while general UGT activity is initially low and rises to adult levels at or around 20 months of age. These data parallel early work performed in animals where the livers of fetal, neonatal and juvenile rabbits and guinea pigs showed the same profile (Lucier et al., 1977).

Our report that total UGT activity may develop by 20 months of age (1.7 years) agrees with previously published data for drugs and compounds metabolized by multiple UGT isoforms (Strassburg et al., 2002). The early development of apparent “maximum” levels of activity may mask the contribution of iso-enzymes that have lower rates of activity and/or develop later. This is because low rates of metabolism performed by one isoform (such as in the picomolar range) can be drowned out by the development of high-capacity isoforms that work, for example; in the micromolar range. For the data presented, this is almost certainly true because although the substrate 4MU is considered non-specific for UGT activity, it is metabolized primarily by the UGT1A family (Burchell et al., 1995). Some members of the UGT2B family including UGT2B4 (Jin et al., 1993), UGT2B15 (Green et al., 1994) and UGT2B7 (Ritter et al., 1990; Jin et al., 1993) have also been reported to metabolise 4MU in vitro although at rates up to 10-fold lower than the UGT1A isoforms.
Despite this, the age at which enzymes reach full adult activities may not be universally indicative of in vivo drug clearance. For example, using allometric scaling intrinsic hepatic clearance of TFP did not appear to reach adult levels until 18.9 years of age, despite UGT being maximally active well before this. In addition, the range of intrinsic clearances calculated was far lower than clinical pharmacokinetic studies with TFP that show clearances of around 500 L/hr (Midha et al., 1988). There is still much discussion over the application and accuracy of the three quarter allometric model, particularly at the individual organ level (Wang et al., 2001). When added to the fact that the parameter for liver blood flow (Q) certainly differs between neonates, children and adults, some uncertainty exists in our model. The underestimation of drug clearance using scaled in vitro UGT data is also an acknowledged problem with this family of enzymes – in contrast to that of the cytochromes P450 (Lin and Wong, 2002).

Our derived $V_{\text{max}}$ and $K_m$ values for UGT 1A4 towards TFP are in good agreement with recently published values which is interesting due to the difference in models (Michaelis-Menten vs. substrate inhibition) used (Uchaipichat et al., 2006a). Inter-laboratory variation, the greater number of human livers assessed by us (27 individual plus a pool of 50 in the current study versus 4 individual livers in the previous study) and differences in sensitivity between our fluorometric assay and HPLC detection employed by Uchaipichat et al. (2006) may also be involved.

Aside from scaling issues, it is not unreasonable for modeled intrinsic clearances to reach apparent adult maxima after full enzyme activity. This may be related to the relative
contribution of different metabolic pathways for TFP. As the contribution of UGT1A4 to total metabolism of TFP is unknown, it is likely that other enzymes metabolize TFP to a greater extent than UGT1A4. Phenothiazine drugs are largely metabolized by CYP2D6 (Llerena et al., 2000) with subsequent phase II metabolism. The major Phase II metabolites commonly reported are sulfate-conjugates (Hartigan-Go et al., 1996) but glucuronide conjugates have also been reported as the major urinary metabolite of phenothiazine drugs (Pieniaszek et al., 1999). The precise metabolic profile for Trifluoperazine is unknown but the contribution of UGT1A4 may not be the deciding factor in total hepatic clearance. Redundancy in metabolic pathways is extremely useful in humans for avoiding toxic consequences - where one pathway may be blocked another simply takes over. However, this also means that study of a single path may not give an accurate picture of whole-body metabolism.

A functional single nucleotide polymorphism in the UGT1A4 isoform recently identified with incidences as high as 9% (Ehmer et al., 2004; Mori et al., 2005). This polymorphism has functional effects towards the anti-cancer drug tamoxifen (Sun et al., 2006) and the atypical anti-psychotic clozapine (Mori et al., 2005). Both of these studies reported apparently higher intrinsic clearances ($V_{\text{max}}/K_{\text{m}}$ ratios) for the polymorphism due to lower $K_{\text{m}}$ values being reported. Although the presence of polymorphisms in UGT1A4 have not been assessed in this study, using the statistical incidence of the polymorphism we would expect that no more than 3 of our samples would contain the polymorphism (corresponding to the highest reported 9% incidence in the German population (Ehmer et al., 2004)).
One of the relative strengths of this study is that our microsomes derive from normal pediatric livers. The only other study to present data on pediatric UGT activity used tissue from patients 2 years of age or less undergoing liver resection for extrahepatic biliary atresia (Strassburg et al., 2002). While most markers appeared normal, the authors could not rule out confounding of their results secondary to liver pathology because it has been demonstrated that UGT mRNA levels can be induced under conditions of acute liver inflammation (Congiu et al., 2002). The main caveat to our pediatric liver tissue is that the post-mortem time of liver collection is unknown. Thus the possibility exists that enzyme activity in our samples had declined from that in live, healthy livers. Despite this, our study presents a valuable addition through our novel UGT1A4 data as well as through being strongly supportive of the earlier paper.

By indicating that drug metabolizing enzyme activity is particularly lacking in infants under 2 years old, our study matches the reported incidences of pediatric adverse drug reactions startlingly well. The rates of adverse drug reaction reported in children show that these are almost 5 times more likely to occur in children under one and 3.5 times more likely in children over one but under four when compared with older children (Menniti-Ippolito et al., 2000). We suggest, based on data presented, that in some cases this is related to a lack of UGT-mediated drug clearance causing systemic accumulation of parent drug and/or reactive metabolites.

Understanding the disposition of drugs in the human body is one of the most fundamental pillars of medicine. Until the development of metabolizing enzymes in childhood is better
understood, prescribing for children will remain problematic. This paper contains the first
description of the development of the UGT1A4 isoform in pediatric liver. Detailed study
of the development of enzyme activity is particularly important for preventing adverse
drug reactions as well as for evaluating and developing new medications for children – a
desperately under-served population in modern medicine.
REFERENCES


CHAPTER 4

THE DEVELOPMENT OF UDP-GLUCURONOSYLTRANSFERASES
1A1 AND 1A6 IN THE PEDIATRIC LIVER

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ABSTRACT

UDP-glucuronosyltransferases (UGTs) are critical for the metabolism and clearance of drugs, chemicals and hormones. The development of UGT1A1 and 1A6 was studied in 50 pediatric liver samples using bilirubin and serotonin activity assays and Western blot as well as pharmacokinetic scaling. UGT activity developed age-dependently in pediatric liver. Maximum activity of 0.7690 nmol/min/mg protein was observed for UGT1A1 at 3.8 months. For UGT1A6, activity matured at 14 months (4.737 nmol/min/mg protein). Protein expression was not age-dependent and activities did not correlate to protein levels for either enzyme. The in vitro activities were used to calculate normalized hepatic clearances using both allometric scaling and the PB/PK model derived from Simcyp® Pediatric. For UGT1A1, allometry predicted normalized adult clearances of 0.0070 L/hr/kg at 3.0 (well-stirred) and 2.8 years (parallel tube), while the Simcyp model showed normalized clearances of 0.0079 L/hr/kg at 2.6 (well-stirred) and 2.5 years (parallel-tube). For UGT1A6, only the Simcyp well-stirred model converged at 0.3524 L/hr/kg at 12.6 months. These data imply independent regulation of UGT1A1 and 1A6 where activity has matured after six months to a year. Total hepatic clearance of substances mediated by these enzymes may mature concurrently, or take longer due to other physiological factors. Late development of UGT enzymes may contribute to chemical, drug and environmental toxicity.
INTRODUCTION

After the cytochromes P450, the UDP-glucuronosyltransferases (UGTs, E.C. 2.4.1.17) are one of the most critical families for clearance and elimination of drugs, endobiotics, dietary compounds, environmental chemicals, and hormones. Despite the importance of these enzymes, published data on their developmental dynamics are scarce (Radominska-Pandya et al., 1999). Notwithstanding this, it is widely believed that the main cause of pediatric adverse drug reactions and chemical toxicity is the ontogenetic lack of drug metabolism. For some metabolizing enzymes, this has been proven to be incorrect, such as for the sulfotransferase and cytochrome P450 3A7 enzymes that are higher in neonates than in children, which may be detoxifying or may form reactive metabolites (Richard et al., 2001; de Wildt et al., 1999). However, evidence suggests that UGTs develop some time after birth and this developmental profile could contribute to negative therapeutic outcomes. Adverse drug reactions are one of the leading causes of pediatric death and illness in the US (Impicciatore et al., 2001) causing approximately 79,000 pediatric hospitalizations per year, of which 31,000 are life threatening or fatal (Miller et al., 2003) with the majority occurring in children under the age of five (Schillie et al., 2009).

Therefore, it is important to dissect the development of individual metabolizing enzymes, including the UGTs, to gain a comprehensive picture of pediatric detoxification capacity.

In the early 1980s, a late-fetal change (30 – 40 weeks of gestation) in hepatic UGT1A1 (from 0.1% to 1.0% of adult activity levels) and post-natal changes that are related to birth age, not gestational age, were reported (Kawade and Onishi, 1981). Subsequently, researchers used biochemical substrates to determine UGT activity in the fetal liver,
demonstrating that bilirubin (UGT1A1) and 1-naphthol (1A1, 1A3, 1A6, 1A7, 1A8, 1A9, 1A10, and 2B7) glucuronidation increase post-natally. Despite these activity differences, similar levels of protein expression were observed between a 13-week neonate and an adult liver. Since neither the antibody nor the substrates were specific (except bilirubin, which was not known at the time), evidence for the maturation of single isoforms was not inferred (Coughtrie et al., 1988). Following this, Burchell et al (1989) demonstrated UGT maturation with fetal and neonatal liver samples from several gestational time points. Similar development of activities to pan-specific substrates were observed except for serotonin (1A4), where adult activities were observed in fetal (16 – 25 weeks) and neonatal liver up to 10 days old (Burchell et al., 1989). More recently, individual UGT isoform development in infants and young children, including two fetal liver samples, were analyzed and showed that pediatric levels of mRNA and protein for UGT isoforms did not differ from adults, but activities were lower up to 2 years of age (Strassburg et al., 2002).

For individual UGT isoforms, postnatal development has been described for UGT1A4 (Miyagi and Collier, 2007) and UGT2B7 (Zaya et al., 2006). Herein, we extend knowledge in this area by investigating the ontogeny of UGT1A1 and 1A6. Within the UGT superfamily, these two enzymes are particularly important as they have the widest tissue distributions, have been extensively studied and possess the broadest substrate affinities. The UGT1A1 isoform is involved in the metabolism and regulation of endobiotics such as estrogen and bilirubin as well as the elimination of several drugs (Williams et al., 2004; Burchell et al., 2000). Polymorphisms in UGT1A1 have functional
consequences that range from Gilbert’s disease, a mild clinical syndrome, to the potentially fatal Crigler-Najjar disease (Nagar and Blanchard, 2006). The UGT1A6 isoform is also involved in drug metabolism and in the clearance of several steroid & thyroid hormones and environmental chemicals (Zhang et al., 2007; Court, 2005; Radominska-Pandya et al., 1999). Similar to 1A1, the 1A6 isoform is also polymorphic with expression and activity affected by genetics, environmental and tissue-specific factors (Bock and Köhle, 2005).

We hypothesized that UGT1A1 and 1A6 would show independent developmental profiles, and would not reach full enzyme activity for several years. Furthermore, based on a combination of enzyme development and physiological development in childhood, we hypothesized that hepatic clearance mediated by these enzymes would likewise show a developmental delay. The goal was to increase our understanding of UGT ontogeny to improve pediatric pharmacology and better understand chemical and environmental toxicities.

**METHODS**

**Materials**

Serotonin, bilirubin, MgCl₂, glycine, 2-pentanone, n-butyl acetate, HCl, ascorbic acid, UDP-glucuronic acid, NaOH, alamethicin (from *Trichoderma viride*), phthalimide, ethyl ether, sodium hypochlorite solution (5.65 – 6%), chloroform-D, sulfuric acid, sodium nitrite, DMSO-D₆, ammonium sulphamate, and ethanol were purchased from Sigma Chemical Company (St. Louis, MO). Serotonin ELISAs were purchased from Rocky
Mountain Diagnostics (Colorado Springs, CO) and ALPCO Immunoassays (Salem, NH). Primary antibodies to UGT1A1 and 1A6 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and secondary antibodies were obtained from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). Western blotting ECL detection system was from GE Healthcare (Buckinghamshire, UK).

**Pediatric and adult liver samples and demographics**

Individual pediatric and pooled adult human liver microsomes were purchased from XenoTech LLC (Lenexa, KS) and Puracyp (Carlsbad, CA) and were derived from post-mortem donors with healthy livers which were acquired through the United Network for Organ Sharing (UNOS) with various causes of death (anoxia, cerebrovascular aneurysm, head trauma, or motor vehicle accident). The samples were processed within 8 hours post-mortem and were free of any infectious disease. Protein content of the microsome samples were provided by the companies. Pediatric microsomes (n = 50) were derived from single livers and in this study the sample population comprised of: Asian (3%), Hispanic (14%), African American (16%), American Indian (3%) and Caucasian (64%) donors, with 15 females and 35 males. Samples ranged from 13 days to 20 years of age, with an average age of 8.0 years. Pooled adult liver microsomes (XTreme 200 Pool, XenoTech LLC) were derived from 200 donors and contained Asian (4%), Hispanic (6%), African American (6%) and Caucasian (84%) ethnicities with equal numbers of men and women (100 each). The average age was 50 with a range of 17 – 78 years.
Synthesis and preparation of ethyl anthranilate

Ethyl anthranilate was synthesized as previously described (Humburger and Hahn, 1972; Staiger and Miller, 1959). Briefly, 0.2 mol phthalimide was dissolved in 110 mL 2 M NaOH solution for 10 minutes at 10 °C then temperature was dropped to 4 °C and the solution left to stand for 16 hours. Subsequently, a solution of 0.2 mol sodium hypochlorite and 0.0183 mol NaOH was added at 10 °C over 5 minutes followed by rapid addition of 0.119 mol sulfuric acid and the compound heated to 35 °C for 30 minutes. The product was filtered and washed with 3x its volume in water, then oven dried at 50 – 55°C for 15 hours. This intermediate (isatoic anhydride) was dissolved in DMSO-D₆ and checked for purity by 1H-NMR, yielding a spectra corresponding to the reference (in ppm): 7.149 (1H, d), 7.259 (1H, t), 7.729 (1H, t), 7.789 (1H, d), 11.713 (1H, s). Upon obtaining acceptable purity, a mixture of the isatoic anhydride with ethanol and NaOH was charged to a round bottom flask in the molar ratio 1:6:0.05. The slurry was stirred and slowly heated to 65 °C and held till bubbling ceased. The organic layer was extracted after being diluted in 3x its volume with water. The final product (ethyl anthranilate) was dissolved in chloroform-D and assessed for purity by 1H-NMR. The final product yielded the following spectra that also corresponded to the reference spectra (in ppm): 1.383 (3H, t), 4.319 (2H, d), 5.751 (2H, s), 6.642 (2H, double of t), 7.259 (1H, t), 7.7887 (1H, d). The compound was subsequently used for the bilirubin diazotization.

For the diazotization, 15 μL of ethyl anthranilate suspended in 1.5 mL of 0.15 M HCl was treated with 45 μL of freshly prepared sodium nitrite (5 mg/mL) then incubated at room temperature for 5 minutes. An aliquot of 15 μL freshly prepared ammonium
sulphamate (10 mg/mL) was added and incubated at room temperature for 3 minutes, after which it was used immediately in the UGT1A1 assay.

**UGT1A1 activity and intrinsic liver bilirubin concentrations**

The activity of UGT1A1 was determined as previously described (Heirwegh et al., 1972). Briefly, 150 μL of 0.4 M Tris-HCl buffer (pH 7.7) containing bilirubin (0.125 mM), UDP-glucuronic acid (5 mM), MgCl₂ (5 mM), alamethicin (50 μg/mg protein), and microsomes (0.5 mg/mL) was incubated for 15 minutes at 37 °C. The reaction was stopped by the addition of 150 μL of 0.4 M glycine-HCl buffer (pH 2.7) and placed on ice for a few minutes. Next, 150 μL of ethyl anthranilate diazo-reagent was added to the mixture and incubated at room temperature for 30 minutes in the dark, then the reaction stopped with 150 μL of freshly prepared 10% ascorbic acid solution (w/v). Bilirubin glucuronides were extracted with a 3x volume of 2-pentanone:n-butyl acetate (17:3, v/v) and frozen at -20 °C. After freezing, samples were thawed, vortexed and centrifuged at 2500 g for 10 minutes. Samples were re-frozen at -20 °C and bilirubin glucuronides detected in the organic phase by absorbance at 530 nm in a Spectramax 340 plus spectrophotometer (Molecular Devices, Sunnyvale, CA). Bilirubin glucuronide concentrations were calculated using the $\varepsilon = 44.4 \times 10^3$ L mol⁻¹ cm⁻¹. Intrinsic levels of bilirubin glucuronide were also determined for each sample as described previously, with the exception that UDP-glucuronic acid was not added. Each sample was read in triplicate. Prior to experimentation, a kinetic time-point assay (at 0, 15, 30, 45, 60, 75, 120 min) was performed to determine the optimal incubation period (15 min). Results were expressed in nmol bilirubin metabolized per minute per mg protein. Bilirubin was
chosen since it is a highly specific substrate for UGT1A1. The intra- and inter-assay CVs for the pooled adult human liver microsomes (positive controls) were 8.5 and 19.0% respectively.

**UGT1A6 activity and intrinsic liver serotonin concentrations**

The activity of UGT1A6 was measured as previously described (Krishnaswamy et al., 2003). Briefly, 100 μL of 0.25 M potassium phosphate buffer (pH 7.5) containing serotonin (100 μM), UDP-glucuronic acid (5 mM), MgCl₂ (5 mM), alamethicin (50 μg/mg protein), and microsomes (0.2 mg/mL) was incubated for 20 minutes at 37 °C. Subsequently, reactions were diluted 50-fold and analyzed with a commercial serotonin ELISA as per the manufacturer's instruction (Alpco Diagnostics, Salem, NH and Rocky Mountain Diagnostics, Colorado Springs, CO). Intrinsic levels of serotonin were also determined for each sample as described above, except UDP-glucuronic acid was not added. Since the Kₘ of serotonin (5.2 – 8.8 mM) is far larger than physiological conditions, a single concentration of 100 μM was used. Since the specificity of the commercial ELISA is < 0.2 % for serotonin metabolites, a substrate depletion approach was used. Specific activity of UGT1A6 was calculated as follows: 100 μM serotonin + intrinsic serotonin - concentration after 20 minute incubation. Each sample was assayed in duplicate. Results were expressed in nmol serotonin metabolized per minute per mg protein. Serotonin was chosen since it is a highly specific substrate for UGT1A6. The average r² of the standard curves from the ELISA kits were 0.983 ± 0.007. The intra- and inter-assay CVs for the pooled adult human liver microsomes (positive controls) were 16.2 and 16.6% respectively.
Western blot for UGT1A1 and 1A6 protein in pediatric liver

The expression of UGT1A1 and 1A6 protein was assessed with Western blot as previously described (Collier et al., 2002). Primary antibodies were purchased from Santa Cruz Biotechnology and were an affinity purified goat polyclonal antibody raised against a peptide mapping near the N-terminus of UGT1A1 or 1A6 of human origin. Briefly, microsomes from individual pediatric livers (10 μg) and pooled adult livers (positive control, 10 μg) were resolved on a 10% SDS-PAGE gel under reducing conditions, transferred to PVDF membranes with semi-dry transfer (Biorad, Hercules, CA) then blocked in PBS-T with 2% non-fat milk powder overnight. Subsequently, membranes were washed and incubated with primary antibody (rabbit anti-UGT1A1/1A6, 1:1000, 2% non-fat milk powder) for 2 hours at room temperature. After primary incubation, membranes were washed again, incubated with a solution of secondary biotinylated donkey anti-rabbit antibody (1:4000, 2% non-fat milk powder, 2% normal donkey serum) for 1 hour at room temperature, washed a further time then incubated with streptavidin-biotinylated HRP (1:3000) for 1 hour. Before detection, membranes were washed a final time and bands were detected on-membrane with TMB substrate for 15 minutes at room temperature. Confirmation of even protein loading was established by staining gels with Coomassie blue. Band intensities were calculated using Image-J software (NIH, Bethesda, MD) and were normalized to the pooled control included on every membrane.

Pharmacokinetics, scaling and statistical analyses

All analyses were performed using GraphPad Prism 5 (La Jolla, CA). Adult levels of enzymatic activity and normalized clearances were determined using a one-phase
exponential association non-linear equation (Equation 4.1), fitted using GraphPad’s ROUT method (Robust non-linear regression and outlier removal). This model assumes that an enzyme will start at some activity at birth and increase with a constant rate of development (K) that fits a curve ending in a maximum rate of action (plateau). \( Y_0 \) represents initial activity after birth and \( Y_{\text{max}} \) represents the max enzyme rate. Activity models were not weighted.

\[
Y = Y_0 + (Y_{\text{max}} - Y_0)(1 - e^{-KX})
\]

**Equation 4.1. One-phase exponential association.**

For analysis, a table of XY coordinates defining the modeled curve was generated and the "age of adult activity/clearance" was defined as the youngest age at which activity reached within 90% of the plateau level (90\(^{\text{th}}\) percentile).

To evaluate hepatic drug clearance, our enzyme kinetic results were modeled using both the well-stirred (Equation 3.1) and the parallel tube equations (Equation 3.2) to calculate adult clearances.

\[
CL_{\text{hepatic}} (L/\text{hr}) = \frac{Q_{\text{hepatic}} \times f_d \times CL_{\text{int}}}{Q_{\text{hepatic}} + f_d \times CL_{\text{int}}}
\]

**Equation 3.1. The well-stirred model.**
Equation 3.2. The parallel tube model.

\[
CL_{\text{hepatic}} \left( \frac{L}{hr} \right) = Q_{\text{hepatic}} \times \left( 1 - e^{-CL_{\text{int}} \times f_u Q_{\text{hepatic}}} \right)
\]

Here, \(Q_{\text{hepatic}}\) is hepatic blood flow, \(CL_{\text{int}}\) is intrinsic clearance and \(f_u\) is the unbound fraction of the drug. Hepatic clearances were generated by using experimental intrinsic clearances \((V_{\text{max}}/K_m)\), assuming a liver size of 1500 g and a hepatic flow rate of 1.5 L/min for adults. The literature \(K_m\) values, that were used to calculate intrinsic clearances, for bilirubin (UGT 1A1) was 5.0 \(\mu\)M (Ciotti et al., 1998) and serotonin (UGT1A6) was 5.2 mM (Krishnaswamy et al., 2003). \(V_{\text{max}}\) was calculated for each sample within the study using experimental enzyme activity, substrate concentrations and Michaelis Menten kinetics (125 \(\mu\)M bilirubin / 1A1 and 100 \(\mu\)M serotonin / 1A6). Since the \(K_m\) for UGT1A6 exceeded physiological concentrations of serotonin, a lower concentration was used that was taken from the linear portion of the Michaelis-Menten curve and was subsequently used to calculate \(V_{\text{max}}\). Microsomal protein per gram liver (MPPGL) was unknown, so the standard variable of 45 mg/g was used (Houston, 1994). Plasma unbound fraction for bilirubin and serotonin were 0.001 (Ostrow et al., 2003) and 0.17 (Breyer-Pfaff et al., 1989), respectively.

Subsequently, an allometric model (Equation 3.3) was used to scale calculated adult clearances (well-stirred and parallel tube) to pediatric clearances using children's weight as the scalar.
Here, \( W_i \) is the weight of the individual and \( W_{\text{std}} \) is the weight of an average adult (20 years of age). Weight of each individual child was used, except for 8 subjects for whom weights were estimated using the 50th percentile for age and gender from the National Center for Health Statistics (2000) growth charts. The average adult weight used was the 50th percentile at 20 years for each gender.

Additionally, a second model derived from Simcyp Pediatric (Sheffield, UK) was used. The model calculated liver size maturation (Equation 4.2), \( Q_{\text{hepatic}} \) (Equation 4.3), MPPGL (Equation 4.4), and \( [P]_{\text{pediatric}} \), the amount of pediatric albumin (Equation 4.5).

The fraction unbound in the pediatric population, \( f_u,_{\text{pediatric}} \), was calculated (Equation 4.6) using adult \( f_u \) and pediatric albumin levels, where \( [P]_{\text{adult}} \) is 44 g/L (McNamara and Alcorn, 2002). Body surface area (in m\(^2\)) was calculated based on height and weight of the individual (Equation 4.7) using a variation of DuBois and Dubois (Wang et al., 1992), where height is in cm and weight is in kg. For the 15 individuals where height and/or weight were missing, values were estimated using the 50th percentile for age and gender from the National Center for Health Statistics (2000) growth charts. The calculated values using the Simcyp equations were then used to calculate hepatic clearance using Equation 2 and 3. Since \( f_u, \) MPPGL, \( Q_{\text{hepatic}} \), and liver size were scaled individually for each donor based on age or body surface area, the allometric model was not applied here.
Liver Size\( (g) = (\text{Body Surface Area})^{1.176} \times 0.722 \)

Equation 4.2. Formula to calculate liver size based on body surface area.

\[
Q_{\text{hepatic}} \ (L/hr) = 0.265 \times 10^{(-0.6492 \times \text{Age} - 1.943 \times \text{Age}^2 + 0.8118 \times \text{Age}^3 + 0.08891 \times \text{Age}^3)}
\]

Equation 4.3. Formula to calculate hepatic blood flow based on age.

\[
MPPGL \ (mg/g) = 10^{(1.407 + 0.0158 \times \text{Age} - 0.000382 \times \text{Age}^2 + 0.0000024 \times \text{Age}^3)}
\]

Equation 4.4. Formula to calculate MPPGL based on age.

\[
[P]_{\text{Pediatric}} \ (g/L) = 1.1287 \times \ln(\text{Age}) + 33.746
\]

Equation 4.5. Formula to calculate pediatric albumin based on age.

\[
\begin{align*}
    f_{u,\text{Pediatric}} &= \frac{1}{1 + \left(1 - f_{u,\text{Adult}}\right) \times \left([P]_{\text{Pediatric}} \right) / \left([P]_{\text{Adult}} \times f_{u,\text{Adult}}\right)}
\end{align*}
\]

Equation 4.6. Formula to calculate pediatric \( f_u \).

\[
\text{Body surface area} \ (m^2) = 0.007184 \times \text{Height}^{0.725} \times \text{Weight}^{0.425}
\]

Equation 4.7. Formula to calculate body surface area.

The goodness of fit for each model was assessed with an F-test, \( r^2 \) values, absolute sums of squares, and standard error of estimates (Sy.x). Both allometric and Simcyp clearance
values were normalized to individual body weights.

RESULTS

UGT1A1 and 1A6 activity

Both UGT isoforms’ activities developed in an age-dependent manner. Data were fit over the entire age range of samples against two different one-phase exponential associations (starting at zero and starting at some number), biphasic (that will model a rise to a peak then fall to a plateau) and sigmoidal equations. F-tests indicated that a one-phase exponential equation starting at some number was the best fit models for both isoforms.

For UGT1A1, the model returned activities of 0.7848 pmol/min/mg protein at birth that increased constantly to apparent maximum adult activities of $0.7690 \pm 0.081$ nmol/min/mg protein (CI 95%: 0.6028 – 0.9351), with the 90th percentile activity (90% of maximum) reached at 3.8 months of age (Figure 4.1a, $r^2 = 0.04748$, absolute sum of squares = 13.52, Sy.x = 0.5363). The model-derived maximum activity for UGT1A1 agrees reasonably with the average rates of bilirubin metabolism measured in pooled (n = 200) adult liver microsomes of $0.58 \pm 0.056$ nmol/min/mg protein.

For UGT1A6, derived activity was 2.560 nmol/min/mg protein at birth that increased to maximum adult activities of $4.737 \pm 0.33$ nmol/min/mg protein (CI 95%: 4.041 – 5.434) with 90% maximum activity occurring at 14 months of age (Figure 4.2a, $r^2 = 0.09244$, absolute sum of squares = 178.8, Sy.x = 1.993). The model fit for UGT1A6 was in excellent agreement with average rates of serotonin metabolism measured in pooled adult
liver microsomes (4.3 ± 0.49 nmol/min/mg protein). Neither UGT1A1 nor 1A6 activities differed significantly with gender or ethnicity.

*UGT1A1 and 1A6 protein expression*

Both UGT1A1 and 1A6 were detected at 56 kDa (on separate Western blots), were visible at all post-natal ages and did not differ significantly with gender or ethnicity. In contrast to activity, UGT1A1 and 1A6 protein levels (determined by area-density analysis compared to an adult standard included on every blot) did not show any age-dependence from 0 – 6 months of age (p = 0.4860 for 1A1 and p = 0.5799 for 1A6), 0 – 12 months of age (p = 0.9831 and p = 0.6769 respectively), 0 – 24 months of age (p = 0.8482 and p = 0.2730 respectively), nor over the entire age range of samples from 13 days to 20 years (p = 0.6589 and p = 0.8455 respectively, Figures 4.1b and 4.2b). Furthermore, protein levels and enzyme activities of UGT1A1 and 1A6 did not significantly correlate for samples from 0 – 6 months of age (p = 0.9593 for 1A1 and p = 0.9522 for 1A6), 0 – 12 months of age (p = 0.9783 and p = 0.7092 respectively), 0 – 24 months of age (p = 0.5118 and p = 0.4661 respectively), nor over the entire age range of samples (p = 0.4104 and p = 0.2193 respectively, Figure 4.1c and 4.2c).
Figure 4.1. The development of UGT1A1 in the pediatric liver.
A: activity towards 125 μM bilirubin increased with age. Model returned an activity of 0.7848 pmol/min/mg protein at birth that increased to apparent maximal adult activity at 0.7690 ± 0.081 nmol/min/mg (CI 95%: 0.6028 – 0.9351). The 90th percentile of activity was reached at 3.8 months of age ($r^2 = 0.04748$, absolute sum of squares = 13.52, Sy.x = 0.5363). Each point represents each sample read in triplicate, ± SEM. The model is unweighted and constrained by medium convergence criteria requiring five consecutive iterations of the fit to change the sum-of-squares by less than 0.0001%. B: protein levels did not correlate with age. Two-tailed Pearson correlation returned $p = 0.6589$ and $r^2 = 0.005801$. C: protein levels did not correlate with enzyme activity. Two-tailed Pearson correlation returned $p = 0.4104$ and $r^2 = 0.02002$. Band intensities were calculated using Image-J software (NIH) and were normalized to the pooled samples on each Western blot.
Figure 4.2. The development UGT1A6 in the pediatric liver.
A: activity toward 100 μM serotonin increased with age. Model returned an activity of 2.560 nmol/min/mg protein at birth and increased to apparent adult activity at 4.737 ± 0.33 nmol/min/mg protein (95% CI: 4.0041 – 5.434). The 90th percentile of adult activity was reached at 14 months of age ($r^2 = 0.09244$, absolute sum of squares = 178.8, $Sy.x = 1.993$). Each point represents each sample assayed in duplicate. The model is unweighted and constrained by medium convergence criteria requiring five consecutive iterations of the fit to change the sum-of-squares by less than 0.0001%. B: protein levels did not correlate with age. Two-tailed Pearson correlation returned $p = 0.8455$ and $r^2 = 0.001132$. C: protein levels did not correlate with enzyme activity. Two-tailed Pearson correlation returned $p = 0.2193$ and $r^2 = 0.04403$. Band intensities were calculated using Image-J software (NIH) and were normalized to the pooled samples on each Western blot.
Pharmacokinetic modeling for UGT1A1 and 1A6

Table 4.1 summarizes the modeled clearance values and goodness of fits for UGT1A1 and 1A6 assessed in both the well-stirred and parallel tube pharmacokinetic models. Interestingly, UGT1A1 showed a higher normalized clearance at birth then dropped to adult clearance levels after a few years. Both the allometric and Simcyp models returned similar maximum hepatic clearances and ages at which normalized adult clearance is reached when raw data was incorporated into the model. After scaling the model results using allometry to account for pediatric clearance, apparent normalized maximum (adult) clearance plateaued at 0.0070 L/hr/kg for both models (well-stirred, Figure 4.3a; parallel tube, Figure 4.3b). The 90th percentile of clearance was reached at 3.0 years (well-stirred) and at 2.8 years (parallel tube). Simcyp models returned similar values to plateau at 0.0079 L/hr/kg (well-stirred, Figure 4.3c; parallel tube, Figure 4.3d). The 90th percentile of clearance was reached at 2.6 years (well-stirred) and at 2.5 years (parallel tube).

Normalized pooled adult clearances were 0.0070 L/hr/kg for allometric scaling and 0.0064 L/hr/kg for Simcyp scaling.

In contrast, UGT1A6 increased to adult levels approximately a year after birth. Both allometric models (Figure 4.4a and 4.4b) and Simcyp parallel tube model (Figure 4.4d) did not converge. The normalized adult clearance for the Simcyp well-stirred model peaked at 0.3524 L/hr/kg (Figure 4.4c). The 90th percentile of clearance was reached at 12.6 months, approximately the same age at which the enzyme matures (14 months). Normalized pooled adult clearances were 0.035 L/hr/kg for allometric scaling and 0.033 L/hr/kg for Simcyp scaling.
Figure 4.3. The development of hepatic bilirubin clearance for UGT1A1 in pediatric liver.
A: the development using the well-stirred model with allometric scaling. Apparent adult clearance plateaued at 0.0070 L/hr/kg. The 90th percentile of adult clearance was reached at 3.0 years of age. B: the development using the parallel tube model with allometric scaling. Apparent adult clearance plateaued at 0.0070 L/hr/kg. The 90th percentile of adult clearance was reached at 2.8 years of age. C: the development using the Simcyp and well-stirred model. The model showed a similar plateau at 0.0079 L/hr/kg. The 90th percentile of adult clearance was reached at 2.6 years. D: the development using the Simcyp and parallel tube model. Clearance plateaued at 0.0079 L/hr/kg. The 90th percentile of adult clearance was reached at 2.5 years. Allometric models are represented by filled circles and solid lines, while Simcyp pediatric models are open circles and dashed lines. Pooled adult clearances are shown as the dotted lines. The model is unweighted and constrained by medium convergence criteria requiring five consecutive iterations of the fit to change the sum-of-squares by less than 0.0001%, with any outlier removed as determined by the residuals.
Figure 4.4. The development of hepatic serotonin clearance for UGT1A6 in pediatric liver.
A: the development using the well-stirred model with allometric scaling. B: the development using the parallel tube model with allometric scaling. C: the development using the Simcyp and well-stirred model. This was the only model to converge. Clearance plateaued at 0.3524 L/hr/kg. The 90th percentile of adult clearance was reached at 12.6 months. D: the development using the Simcyp and parallel tube model. Allometric models are represented by filled circles, while Simcyp pediatric models are open circles and dashed lines. Pooled adult clearances are shown as the dotted lines. The model is unweighted and constrained by medium convergence criteria requiring five consecutive iterations of the fit to change the sum-of-squares by less than 0.0001%, with any outlier removed as determined by the residuals.
Table 4.1. Summary table for modeled bilirubin and serotonin clearance values.

<table>
<thead>
<tr>
<th>UGT1A1 Clearance</th>
<th>Parameter</th>
<th>Well-Stirred</th>
<th></th>
<th>Parallel Tube</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Allometric</td>
<td>Simcyp</td>
<td>Allometric</td>
<td>Simcyp</td>
</tr>
<tr>
<td>Normalized Adult Clearance</td>
<td></td>
<td>0.0070</td>
<td>0.0079</td>
<td>0.0070</td>
<td>0.0079</td>
</tr>
<tr>
<td>R²</td>
<td></td>
<td>0.0131</td>
<td>0.0077</td>
<td>0.0131</td>
<td>0.0076</td>
</tr>
<tr>
<td>95% CI of Adult Clearance</td>
<td></td>
<td>0.0058, 0.0082</td>
<td>0.0063, 0.0095</td>
<td>0.0058, 0.0082</td>
<td>0.0063, 0.0095</td>
</tr>
<tr>
<td>Age within 90% of Adult</td>
<td></td>
<td>3.0 yrs</td>
<td>2.6 yrs</td>
<td>2.8 yrs</td>
<td>2.5 yrs</td>
</tr>
<tr>
<td>Weighted Sum of Squares</td>
<td></td>
<td>0.0034</td>
<td>0.0058</td>
<td>0.0035</td>
<td>0.0059</td>
</tr>
<tr>
<td>Sy.x</td>
<td></td>
<td>0.0049</td>
<td>0.0064</td>
<td>0.0049</td>
<td>0.0064</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>UGT1A6 Clearance</th>
<th>Parameter</th>
<th>Well-Stirred</th>
<th></th>
<th>Parallel Tube</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Allometric</td>
<td>Simcyp</td>
<td>Allometric</td>
<td>Simcyp</td>
</tr>
<tr>
<td>Normalized Adult Clearance</td>
<td></td>
<td>Not converged</td>
<td>0.3524</td>
<td>Not converged</td>
<td>Ambiguous</td>
</tr>
<tr>
<td>R²</td>
<td></td>
<td>-</td>
<td>0.0686</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>95% CI of Adult Clearance</td>
<td></td>
<td>-</td>
<td>0.3149, 0.3900</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Age with 90% of Adult</td>
<td></td>
<td>-</td>
<td>12.6 mo.</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Weighted Sum of Squares</td>
<td></td>
<td>-</td>
<td>2.182</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Sy.x</td>
<td></td>
<td>-</td>
<td>0.1548</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Clearances are expressed as L/hr/kg.
DISCUSSION

The major finding here is that UGT1A1 and 1A6 activities develop at different rates in the pediatric liver. The results for UGT1A1 are similar to, and supported by, published studies into UGT1A4 (Miyagi and Collier, 2007) and 2B7 (Zaya et al., 2006). The *in silico* models used indicate that UGT1A1 enzyme activity is present, but very low at birth and increases to a plateau after 3.8 months. In contrast, UGT1A6 has comparatively higher activity at birth and increased to adult levels at 14 months. Both findings are consistent with previous laboratory and clinical studies (Burchell et al., 1989; Kawade and Onishi, 1981). Despite activity differences, age-related changes in UGT1A1 and 1A6 proteins were not observed and enzyme activities did not correlate with protein levels. Again, this is similar to an earlier study demonstrating that UGT1A1 and 1A6 had similar mRNA and protein expression between toddlers (6 – 24 months) and adults, but significantly different glucuronidation activities for several pan-specific substrates (Strassburg et al., 2002). Interestingly, these results in UGT1A isoforms contrast with the current knowledge regarding ontogeny of UGT2B, where for UGT2B7 protein expression and activity correlate (Zaya et al., 2006).

Aside from UGTs, similar findings have been reported for other pediatric liver enzymes including cytochromes P450 2C9, 2C19 and 3A4 (Hines, 2007) and carboxylesterases 1 and 2 (Pope et al., 2005). Many of these Phase I enzymes appear to be transcriptionally regulated. However, since UGT1A enzyme activity and protein expression did not correlate with each other herein nor in a previous report (Strassburg et al., 2002), it is possible that post-translational modification regulates UGT ontogeny. Comparatively
little is known about the structure-function relationships of UGTs compared to other metabolizing enzymes, in part because a crystal structure has not yet been elucidated. One important study shows that a specific glycosylation site on UGT2B15 and 2B20 is critical for modulating activity (Barbier et al., 2000). A similar site/mechanism and associated developmental signal may account for changes in other isoforms’ catalytic activity after birth, such as those observed herein.

Alternatively, it was demonstrated that adenosine-related nucleotides inhibit rat UGT activity, an allosteric mechanism that may also be relevant for developmental signaling (Nishimura et al., 2007). There is further support for this mechanism in humans since the naturally occurring soy compound daidzein has been shown to allosterically increase human hepatic UGT1A1 activity (Pfeiffer et al., 2005). Allosteric signaling would allow UGT protein expression to remain steady throughout childhood, but enzyme activity to vary based on intracellular development and/or levels of signaling molecules.

An acknowledged limitation of this study is that the data herein are derived from only post-natal samples. Despite this, some evidence that UGT activity exists at birth for both UGT1A1 and 1A6 is presented since our models do not converge to zero. Rather, the modeled activities indicate that UGT1A1 at birth may be very low (in the order of 1/1000 of adult activity) or, for 1A6, more than 50% of adult activity levels and this is consistent with previous studies (Burchell et al., 1989). Therefore, it would be useful for future work to determine the molecular mechanisms behind enzyme maturation to adult activities since purely transcriptional activation seems unlikely.
Going beyond enzymology, when UGT1A6 hepatic clearances were scaled and normalized, maximum clearance did not occur until a year of age. This coincides with the age of enzyme maturation at 14 months. Although, the modeled UGT1A1 clearance was higher at birth and decreased to adult levels after 2 – 3 years of age, this should be interpreted with caution since the results are in contrast to enzyme maturation and may be an artifact of our small sample size in the age ranges from 0 to 6 months. Additionally, the regression used may not accurately model the fast maturation of UGT1A1 with so few samples, so correct interpretation could also be that full clearance capacity develops almost immediately after birth.

Because most chemicals are cleared by multiple enzymes, studying a single enzyme may not give an accurate picture of whole body metabolism and clearance. For example, acetaminophen is metabolized by UGT1A1, 1A6 and 1A9 and sulfotransferases directly (95% of metabolism) and also by cytochromes P450 followed by sulfotransferases and glutathione-S-transferases (5% of clearance). For acetaminophen, clearance matures at approximately 2 years of age, due to inherent redundancy and promiscuity in hepatic metabolism (Anderson and Holford, 2009). Despite this, the data presented may be clinically significant for drugs primarily or exclusively metabolized by a single enzyme. This is a clinical issue for pediatric drug administration, such as the use of morphine (that is metabolized only by UGT1A3 and 2B7) in neonates (Knibbe et al., 2009). A caveat to making absolute statements regarding UGT development and clinical clearances derived from this work is that, although scaling for cytochromes P450 activities from in vitro to whole body clearance has been relatively accurate, scaling of UGT data is usually vastly
under-predictive (Miners et al., 2006). Hence, *in vitro* scaling attempts should be compared to clinical observations for correctness and also to develop a greater accuracy and predictivity of UGT scaling.

With regard to our scaling approaches, the allometric model to $\frac{3}{4}$ power scaling was chosen since it has been shown that this is generally superior to scaling using body surface area (Anderson and Holford, 2009). Moreover, the Simcyp model was used because of our desire to use a physiologically-based approach to pharmacokinetic scaling and compare this to purely mathematical pharmacokinetics. Of the several commercial models that exist, Simcyp is the one we have available and has been used in the past to evaluate pediatric scaling (Johnson et al., 2006). Comparison of the models is important to assess whether the PB/PK approach is superior for all data types and to refine mathematical algorithms.

The current paper also makes an interesting contribution to existing knowledge since a maturational component has been added. This maturation component cannot be described by allometry alone when predicting pediatric clearances from adult data (Anderson and Holford, 2009). Increases in enzyme activity per gram of microsomal protein have been reported herein and elsewhere and at least one author has postulated an increase in MPPGL (Barter et al., 2008). This seems sensible, although the developmental mechanisms of this, and indeed its occurrence, have not been well measured. Although small, this is the first report of the development of UGT1A1 and 1A6 in normal pediatric livers from the neonate (13 days) to adult (20 years of age) that also includes scaled,
normalized hepatic clearances and comparisons of PK and PBPK models. The findings presented support and are supported by previous publications that have taken a more general approach using pan-specific antibodies and biochemical substrates. Furthermore, positive controls in these experiments utilized pooled adult microsomes from 200 human donors to define "adult" levels of activity, a useful and robust internal control since inter-individual variation in glucuronidation activity can be high. In addition, because the pooled adult samples contained equal number of males and females and ranged from 17 to 78 years, gender and age biases in the “positive adult control” were minimized, including any differential hormonal effects on enzyme expression and activities. This is similar for the pediatric liver samples assessed that contained 15 female and 35 males across all age ranges. Finally, although we did not specifically determine polymorphisms of UGT1A1 or 1A6 in these samples, the calculated clearances presented will be inclusive of polymorphic variations and true estimates of the population variability.

In summary, this study is not only important from a basic biology/pharmacology standpoint, but also from medical, environmental and regulatory perspectives. Medications are already prescribed that have not been adequately studied for safety in pediatric medicine and as diseases of childhood continue to move from acute to chronic etiologies, this will only increase. Additionally, regulatory bodies are increasingly requested to raise pediatric exposure limits and safety levels for environmental chemicals, commonly without good human data. Studies such as this can contribute by describing pediatric development and also by providing a relevant framework for the understanding of developmental physiology and pharmacology. This approach can improve the
understanding of childhood development, environmental safety and pediatric medicine.
REFERENCES


CHAPTER 5

THE NEONATAL DEVELOPMENT OF HEPATIC UGT1A9:
IMPLICATIONS OF PEDIATRIC PHARMACOKINETICS.

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ABSTRACT

This paper reports on the development of UDP glucuronosyltransferase 1A9 (UGT1A9) in neonatal and pediatric liver. The substrate 4-methylumbelliferone (4MU) with specific inhibition by niflumic acid was used to define specific UGT1A9 activity. Subsequently, in silico pharmacokinetic (PK) and physiologically-based pharmacokinetic (PBPK) modeling was used to determine maturation of UGT1A9 and hepatic clearance. One-phase exponential association modeled maximal enzyme activity was 27.94 nmol/min/mg protein at 4 months of age, which had high concordance with the average \( V_{\text{max}} \) in 45 individual adult (>20 years) livers of 28.98 nmol/min/mg protein. The activity of UGT1A9 ranged 7.5-fold in the adult population (4.1 – 54.5 nmol/min/mg protein). Expression of the 1A9 protein increased with postnatal age and activity correlated to protein expression in neonates (Spearman \( r = 0.84 \)). Furthermore, scaling intrinsic hepatic clearance of 4MU with an allometric PK model yielded a high clearance at birth, falling to adult levels (1.12 l/hr/kg at 18.1 years for well-stirred or 1.23 l/hr/kg at 18.7 years for parallel tube). The Simcyp PBPK model returned low clearances at birth that increased to adult levels of 0.97 l/hr/kg at 1.3 months (well-stirred) and 1.11 l/hr/kg at 1.0 months (parallel tube). Neither UGT activities nor hepatic clearances differed with gender or ethnicity. The UGT1A9 isoform seems to have very high capacity for xenobiotic substrates which may explain why UGT1A9 substrates, such as propofol, have higher clearances in children than in adults.
INTRODUCTION

The UDP-glucuronosyltransferase (UGT, E.C. 2.4.1.17) super-family is critical for the metabolic clearance of most endobiotics and xenobiotics (Radominska-Pandya et al., 1999). The superfamily is divided into UGT1A, UGT2A, UGT2B, and UGT3A subfamilies in humans containing nine, three, seven, and two isoforms, respectively (Radominska-Pandya et al., 1999). Of the known UGT isoforms, twelve are expressed in the adult human liver although substrates (either endobiotic or xenobiotic) for three of these (UGT1A3, 2B10 and 2B11) have not been identified (Radominska-Pandya et al., 1999; Guillemette et al., 2010). The UGTs are generally promiscuous with high capacity, although specificity of individual isoforms does occur. For example, UGT1A9 is the sole pathway for propofol glucuronidation (Rowland et al., 2008) and the predominant route for mycophenolic acid (Mackenzie, 2000) and SN-38 glucuronidation (Villeneuve et al., 2003).

Within the UGT1A subfamily, UGT1A9 is expressed in multiple tissues including the liver, intestines, and kidneys (Strassburg et al., 1999) and may be present in the placenta (unpublished). Besides being the specific or major pathway for several drugs, it is also important in the metabolism of planar phenols, bile acids, fatty acids, hormones, steroids, dietary compounds and tobacco pro-carcinogens (Radomska-Pandya et al., 1999; Bock, 2010; Tsoutsikos et al., 2004). Although functional polymorphisms in UGT1A9 are not common, the major allelic variant UGT1A9*3/*3, confers very low activity in approximately 4% of the population and is problematic for patients undergoing cancer treatment using irinotecan/SN-38 (Villeneuve et al., 2003).
The UGT1A9 isoform is absent from fetal liver (Strassburg et al., 2002), and the post-natal development of hepatic UGT1A9 has not been reported. Current knowledge of UGT development shows that general activity (using substrates metabolized by multiple isoforms) develops at the same rate in the first six months of age, regardless of the gestational age at birth and that mRNA for all hepatic UGTs are expressed within this same timeframe (Strassburg et al., 2002; Onishi et al., 1979; Kawade and Onishi, 1981). More recently, data from our laboratory and others’ have shown that individual UGT isoforms develop independently in neonatal and pediatric liver with UGT1A1, 1A4 and 2B7 being very low or absent and UGT1A6 possessing up to 50% of adult activities at birth (Miyagi and Collier, 2007; Miyagi and Collier, 2011; Zaya et al., 2006).

Although it is well recognized that children have altered drug disposition, knowledge of drug metabolism and pharmacokinetics in the pediatric population is sparse (Anderson, 2010). Despite this, children are commonly switched from pediatric to adult dosing schedules at or around 12 years of age, leading to assumptions that drug metabolism and clearance reaches adult levels during early childhood. In fact, while dosing is reduced for some drugs, in several cases pediatric doses are higher than those administered to adults (Vandermeersch et al., 1989). Prior development of pediatric dosing schedules has been largely empirical and on the (incorrect) assumption that children are just small adults, but to better serve the pediatric population, it is essential to understand biochemical and physiological development of the liver and hence unique pharmacokinetics in children. Such studies will help to guide more appropriate dosing, prevent adverse drug and chemical reactions and improve drug development in pediatric medicine.
We hypothesized that UGT1A9 develops before the nominal age of adulthood (21 years). To test this, the isoform’s activity was determined in sub-cellular fractions (microsomes) from 50 normal pediatric liver samples (0 - 20 years of age) and 45 adult liver samples (21 – 78 years of age) with the positive control being UGT1A9 activity in a pool of adult liver microsomes (n = 200). The general UGT substrate 4-methylumbelliferone (4MU) with and without the UGT1A9-specific inhibitor niflumic acid (NFA) was used for activity studies (Miners et al., 2011). Using a classical two-stage pharmacokinetic approach, we employed both allometric (PK) and physiologically-based pharmacokinetic (PBPK) models to determine maturation of UGT1A9, estimate hepatic clearance from in vitro data and assess population variability.

MATERIALS AND METHODS

Materials

Bicinchoninic acid kit, bovine serum albumin, 4-methylumbelliferone sodium salt, alamethicin (from *Trichoderma viridae*), MgCl₂, Tris, and uridine diphosphoglucuronic acid were purchased from Sigma Chemical Company (St Louis, MO). Niflumic acid was from MP Biomedicals, LLC (Solon, OH). Secondary antibodies were purchased through Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). Streptavidin-horseradish peroxidase was purchased through GE Healthcare (Buckinghamshire, UK). Pierce ECL Western Blotting Substrate was purchased through Thermo Scientific, Inc. (Rockford, IL).
Pediatric and Adult Liver Samples and Demographics

Pediatric and adult liver microsomes were purchased from Xenotech, LLC (Lenexa, KS), Puracyp, Inc. (Carlsbad, CA), and Life Technologies, Corp. (Carlsbad, CA). All tissues were derived from post-mortem donors with healthy livers which were acquired through the United Network for Organ Sharing with various causes of death (anoxia, cerebrovascular aneurysm, head trauma, or motor vehicle accident). The samples were processed within 8 hours post-mortem and were free of any infectious disease. Protein content of the microsome samples were provided by the companies. Eight samples were processed from individual S9 fractions, which were separated into cytosol and microsomes by centrifuging at 100,000g for one hour (Collier et al., 2002). Microsome pellets were re-suspended in 0.1 M Tris buffer with 5 mM MgCl$_2$ at pH 7.4, and protein content was assayed by the bicinchoninic acid method using bovine serum albumin as a standard (Smith et al., 1985). Pediatric and adult demographics are shown in Table 5.1.

Table 5.1. Summary table for sample demographics.

<table>
<thead>
<tr>
<th></th>
<th>Pediatric (50)</th>
<th>Adult (45)</th>
<th>Xtreme (200)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethnicity % (n)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>African American</td>
<td>12 (6)</td>
<td>2 (1)</td>
<td>4.5 (9)</td>
</tr>
<tr>
<td>American Indian</td>
<td>2 (1)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Asian</td>
<td>2 (1)</td>
<td>2 (1)</td>
<td>1.5 (3)</td>
</tr>
<tr>
<td>Caucasian</td>
<td>66 (33)</td>
<td>87 (39)</td>
<td>86 (172)</td>
</tr>
<tr>
<td>Hispanic</td>
<td>12 (6)</td>
<td>9 (4)</td>
<td>8 (16)</td>
</tr>
<tr>
<td>Sex % (n)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Females</td>
<td>32 (16)</td>
<td>38 (17)</td>
<td>50 (100)</td>
</tr>
<tr>
<td>Males</td>
<td>68 (34)</td>
<td>62 (28)</td>
<td>50 (100)</td>
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<tr>
<td>Age Range</td>
<td>13 days - 20 years</td>
<td>21 - 78 years</td>
<td>11 - 83 years</td>
</tr>
<tr>
<td>Average Age</td>
<td>8.0 years</td>
<td>50 years</td>
<td>48 years</td>
</tr>
</tbody>
</table>
**Western Blot for Hepatic UGT1A9**

The expression of UGT1A9 was assessed with western blot as previously described (Sutherland et al., 1992). Primary antibodies were generated by multiple antigenic peptide technology (Posnett et al., 1988) using the sequence SNCRSLFKDKKLVEYLKES. Peptide sequence antigenicity was determined using JaMBW Antigenicity plot ([www.bioinformatics.org/JaMBW/3/1/7](http://www.bioinformatics.org/JaMBW/3/1/7)). Lack of sequence homology to other UGT isoforms was evaluated with ClustalW ([www.ebi.ac.uk/clustalw](http://www.ebi.ac.uk/clustalw)) and NCBI BLAST. A sheep was inoculated with 0.3 mg of peptide and four immunisations were administered four weeks apart with bleeds being taken after the 2nd, 3rd and 4th immunisations. Peptide synthesis was carried out by AltaBioscience (Birmingham, UK) and antibody production by Diagnostics Scotland (now Alba Bioscience, Edinburgh, UK). Microsomes from individual pediatric livers (5 μg) and pooled adult livers (positive control, 5 μg) were resolved on a 10% SDS-PAGE gel under reducing conditions, transferred to PVDF membranes with semi-dry transfer (Biorad, Hercules, CA) then blocked in Tris-buffered saline with 0.1% Tween-20 at pH 9.0 (TBS-T) containing 5% non-fat milk powder overnight. Subsequently, membranes were washed and incubated with primary antibody (sheep anti-UGT1A9, 1:2000, TBS-T with 5% non-fat milk) for two hours at room temperature. After primary incubation, membranes were washed with TBS-T, incubated with biotinylated donkey-anti-goat antibody (1:10,000, TBS-T with 5% non-fat milk powder) for one hour at room temperature, washed a further time then incubated with streptavidin-biotinylated horseradish peroxidase (1:15,000, TBS-T) for one hour. Before detection, membranes were washed a final time and bands were detected with ECL reagent for 30 minutes. Confirmation of even protein loading
was established by staining gels with Coomassie blue. The intensities of protein bands on membranes were calculated using Image-J software (NIH, Bethesda, MD) and were normalized to the pooled control included on every membrane. Bands were expressed in relative density units.

**UGT 1A9 activity with 4MU and NFA**

The activity of UGT1A9 was determined using the general UGT substrate 4MU (at 100 µM) in the presence and of the UGT1A9-specific inhibitor NFA at 2.5 µM (Miners et al., 2011) as previously described by us (Collier et al., 2000) except that alamethicin (50 µg/mg protein) was used as the activator. Specific UGT1A9 metabolism was calculated as follows: [(rate with 4MU – (rate with 4MU + NFA))]. Reactions were carried out in a 96-well microplate and detected using a Gemini XS microplate fluorometer (Molecular Devices, Sunnyvale, CA). Results were transformed to pmol/min/mg protein using a standard curve generated with 4MU ($r^2 = 0.9994 \pm 0.009$). The intra- and inter-assay CV for the pooled adult human liver microsomes (positive controls) were 15.9% and 18.3% respectively. Each sample was assayed in triplicate.

**Pharmacokinetics, scaling and statistical analyses**

A two-stage approach to estimating population pharmacokinetics was undertaken. Adult levels of enzymatic activity and normalized clearances were determined using a one-phase exponential association non-linear equation (Equation 4.1), fitted using GraphPad’s robust non-linear regression and outlier removal. This model assumes that an enzyme will start at some activity at birth and increase with a constant rate of development (K)
that fits a curve ending in a maximum rate of action (plateau). The value $Y_0$ represents initial activity after birth (constrained so $Y_0 \geq 0$) and $Y_{\text{max}}$ represents the maximum rate of the enzyme reaction. Activity models were not weighted.

$$Y = Y_0 + (Y_{\text{max}} - Y_0)(1 - e^{-kY})$$

**Equation 4.1. One-phase exponential association.**

For analysis, a table of XY coordinates defining the modeled curve was generated and the "age of adult activity/clearance" was defined as the youngest age at which activity reached within 90% of the plateau level (90th percentile).

The compound NFA exhibits mixed (competitive and non-competitive) inhibition for UGT1A9 and using Michaelis-Menten kinetics to scale for $V_{\text{max}}$ is not appropriate. Instead the two-site model is used (Miners et al., 2011):

$$V = \frac{V_{\text{max}} \times [S]}{K_m (1 + [I]/K_i') + [S](1 + [I]/K_i)}$$

**Equation 5.1. Two-side binding model.**

In Equation 5.1, $K_i$ (0.11) and $K_i'$ (0.30) represent the inhibitor constants for the EI and ESI complexes, respectively (Miners et al., 2011). Here, [S] is the substrate concentration (4MU, 100 μM) and [I] is the inhibitor concentration (NFA, 2.5 μM). The literature value for the Michaelis constant, $K_m$, is 8.0 μM (Uchaipichat et al., 2004).
Auto-activation, the phenomenon whereby clearance of substrate is concentration-dependent, has been observed for UGT1A9 and 4MU (Uchaipichat et al., 2004). When auto-activation occurs, the intrinsic clearance \( (\text{CL}_{\text{int}}) \), traditionally derived by \( V_{\text{max}}/K_{\text{m}} \), is not appropriate. The alternative term “maximum clearance” \( (\text{CL}_{\text{max}}) \) has been described and is used to provide the greatest clearance possible when the enzyme is fully activated, before saturation (Houston and Kenworthy, 2000):

\[
\text{CL}_{\text{max}} = \frac{V_{\text{max}} \times (n-1)}{S_{50} \times n(n-1)^{\frac{1}{n}}}
\]

**Equation 5.2. Formula for maximum clearance when there is auto-activation.**

In the equation above (Equation 5.2), the value of \( n \) for UGT1A9/4MU is 2.2 and the \( S_{50} \) value is 8.0 \( \mu \)M, both were originally derived in recombinant human UGT1A9 in transfected HEK293 cells (Uchaipichat et al., 2004).

To evaluate hepatic drug clearance, our enzyme kinetic results were modeled using both the well-stirred (Equation 3.1) and the parallel tube equations (Equation 3.2), modified to replace intrinsic clearance \( (\text{CL}_{\text{int}}) \) with \( \text{CL}_{\text{max}} \).

\[
\text{CL}_{\text{hepatic}} \ (L/\text{hr}) = \frac{Q_{\text{hepatic}} \times f_{u} \times \text{CL}_{\text{max}}}{Q_{\text{hepatic}} + f_{u} \times \text{CL}_{\text{max}}}
\]

**Equation 3.1. The well-stirred model.**
\[ \text{CL}_{\text{hepatic}} (L/hr) = Q_{\text{hepatic}} \times \left( 1 - e^{-\frac{-CL_{\text{max}} \times f_u}{Q_{\text{hepatic}}}} \right) \]

**Equation 3.2. The parallel tube model.**

Here, \(Q_{\text{hepatic}}\) is hepatic blood flow. Hepatic clearances were generated by using Equation 2, assuming a liver size of 1500 g and a hepatic flow rate of 1.5 L/min for adults.

Microsomal protein per gram liver (MPPGL) was unknown, so the standard variable of 45 mg/g (Houston, 1994) was used for adults and in the allometric model. Since no values for 4MU plasma unbound fraction exists, the \(f_u\) for HEK293 cell lysate was 0.95. However, 4MU binds to human serum albumin (HSA). In an HEK293 lysate containing 2% HSA, \(f_u\) decreased to 0.14. Since this is more physiologically relevant, this value will also be used instead (Rowland et al., 2007). Furthermore, previous experiments show the “albumin” effect on \(V_{\text{max}}\) is negligible, with increases less than 3% in presence of HSA (0.1%, 1.0%, and 2.0%) versus incubations without HSA (Rowland et al., 2008).

Subsequently, an allometric model (Equation 3.3) was used to scale calculated adult clearances (well-stirred and parallel tube) to pediatric clearances using children's weight as the scalar.

\[ \text{CL}_{\text{pediatric}} (L/hr) = CL_{\text{hepatic}} \times \left( \frac{W_i}{W_{\text{std}}} \right)^{3.3} \]

**Equation 3.3. The allometric model.**

Here, \(W_i\) is the weight of the individual and \(W_{\text{std}}\) is the weight of an average adult (20
years of age). Weight of each individual child was used, except for 8 subjects for whom weights were estimated using the 50th percentile for age and gender from the National Center for Health Statistics (2000) growth charts. The average adult weight used was the 50th percentile at 20 years for each gender. Allometric scaling was also used for individual adults if they differed from the average adult weight.

Additionally, a second PBPK model derived from Simcyp Pediatric (Sheffield, UK) was used (Miyagi and Collier, 2011). The model calculated liver size maturation (Equation 4.2), $Q_{\text{hepatic}}$ (Equation 4.3), $MPPGL$ (Equation 4.4), and $[P]_{\text{pediatric}}$, the amount of pediatric albumin (Equation 4.5). The fraction unbound in the pediatric population, $f_{u,\text{pediatric}}$, was calculated (Equation 4.6) using adult $f_u$ and pediatric albumin levels, where $[P]_{\text{adult}}$ is 44 g/L (McNamara and Alcorn, 2002). Body surface (in m²) was calculated based on height and weight of the individual (Equation 4.7) using a variation of DuBois and Dubois (Wang et al., 1992), where height is in cm and weight is in kg. For the 15 individuals where height and/or weight were missing, values were estimated using the 50th percentile for age and gender from the National Center for Health Statistics (2000) growth charts. The calculated values using the Simcyp equations were then used to calculate hepatic clearance using Equation 3 and 4. Since $f_u$, $MPPGL$, $Q_{\text{hepatic}}$, and liver size were scaled individually for each donor based on age or body surface area, the allometric model was not applied here.

$$\text{Liver Size (g)} = (\text{Body Surface Area})^{1.176} \times 0.722$$

**Equation 4.2. Formula to calculate liver size based on body surface area.**
\[ Q_{\text{hepatic}} \ (L/hr) = 0.265 \times 10^{(-0.6492 + 1.943 \times \text{Age} - 0.8118 \times \text{Age}^2 + 0.08891 \times \text{Age}^3)} \]

**Equation 4.3. Formula to calculate hepatic blood flow based on age.**

\[ MPPGL \ (mg/g) = 10^{(1.407 + 0.0158 \times \text{Age} - 0.000382 \times \text{Age}^2 + 0.0000024 \times \text{Age}^3)} \]

**Equation 4.4. Formula to calculate MPPGL based on age.**

\[ [P]_{\text{Pediatric}} \ (g/L) = 1.1287 \times \ln(\text{Age}) + 33.746 \]

**Equation 4.5. Formula to calculate pediatric albumin based on age.**

\[
\frac{1}{f_u,\text{Pediatric}} = \frac{1}{\left[1 + \left(\frac{1 - f_{u,\text{Adult}} \times [P]_{\text{Pediatric}}}{[P]_{\text{Adult}} \times f_{u,\text{Adult}}}\right)\right]} 
\]

**Equation 4.6. Formula to calculate pediatric f_u.**

\[ \text{Body surface area} \ (m^2) = 0.007184 \times \text{Height}^{0.725} \times \text{Weight}^{0.425} \]

**Equation 4.7. Formula to calculate body surface area.**

For adults, liver size, \(Q_{\text{hepatic}}\), and MPPGL were determined, but \(f_u\) were set to their adult levels at 0.14. Goodness of fits for each model were assessed with an F-test, \(r^2\) values, absolute sums of squares, and standard error of estimates (Sy.x). Both allometric and Simcyp clearance values were normalized to individual body weights. All analyses were performed using GraphPad Prism 5 (La Jolla, CA).
RESULTS

UGT1A9 Activity

The activity of UGT1A9 developed in an age-dependent manner. Data were fit over the age range of pediatric samples (13 days to 20 years of age) against one-phase exponential association (starting at number greater than or equal to zero), biphasic (that will model a rise to a peak then fall to a plateau), and sigmoidal equations. Comparisons of models using the F-test indicated that a one-phase exponential equation was the best fit model. The model reported zero activity at birth, increasing to a maximum plateau of $27.94 \pm 1.519$ nmol/min/mg protein (CI 95%: 24.96 – 30.92) at approximately 4.0 months of age (Figure 5.1). The average of all 45 individual adult clearances was $28.98 \pm 1.789$ nmol/min/mg protein, which is in excellent agreement with the model-derived peak pediatric activity. The model-derived maximum activity also has reasonable concordance with the average rate of metabolism from pooled adult liver microsomes ($39.23 \pm 0.892$ nmol/min/mg of protein). Adult values for 4MU metabolism ranged 7.5-fold (4.1 – 54.5 nmol/min/mg protein) and activities did not differ with gender or ethnicity (data not shown).
Figure 5.1. The development of UGT1A9 activity in the pediatric liver.
Apparent maximum adult activity is reached at 4.0 months with apparent mean adult activity being $27.94 \pm 1.519 \text{ nmol/ min/mg protein}$. Range for all samples was $0 – 81.46 \text{ nmol/min/mg protein}$ and range of adult (>20 years) was $4.1 – 54.5 \text{ nmol/min/mg protein}$. The model is fit from ages 0 to 20 years, un-weighted and constrained by medium convergence criteria requiring five consecutive iterations of the fit to change the sum-of-squares by less than 0.0001%. The dashed line represents the pooled sample ($n = 200$) while the dotted line is the averaged individual adult samples ($n = 45$).

**UGT1A9 Protein Expression**

The UGT1A9 protein was detected at 65 kDa (Figure 2A). Protein was visible at all post-natal ages and did not differ significantly with gender or ethnicity. The protein expression of UGT1A9 correlated with age for children under one year ($n = 11$, $P = 0.002$, Spearman $r = 0.84$) with expression reaching adult levels at 10.0 months of age (Figure 2B).

Furthermore, enzyme activities of UGT1A9 correlate significantly to amounts of protein in those same samples under one year of age ($P = 0.025$, Spearman $r = 0.68$, Figure 2C). A 12-fold range in protein expression was observed in adults ($13 – 155$ density units).

The experimental average of $49.40 \pm 1.02$ density units was in excellent agreement with the modeled plateau of $58.04 \pm 5.9$ density units.
An additional sub-set of microsomal samples (n = 8) were derived by differential centrifugation in-house from commercially-supplied S9 fractions. Despite being diluted to the same concentration as all other samples and despite returning similar activities, these eight samples consistently returned significantly higher protein expression when assessed by western blot. The average density units were 539 ± 136 (range 411 – 831) and this corresponds to approximately 11–fold greater apparent protein expression in the subset of samples derived this way. These samples were not included in our analyses.

**Pharmacokinetic Modeling for UGT1A9**

The modeled clearance values and goodness of fits for UGT1A9 in both the well-stirred and parallel tube PK models are shown in Table 5.2. Interestingly, the allometric models showed a higher initial normalized clearance then decreased to adult levels. Apparent normalized adult clearance plateaued at 1.12 ± 0.07 l/hr/kg and 1.23 ± 0.07 l/hr/kg, for well-stirred and parallel tube models respectively (Figure 3A and 3B). The adult clearance was reached at 18.1 years (well-stirred) and at 18.4 years (parallel tube). The Simcyp models showed opposite results; initial clearance values were low and increased to adult levels and returned similar plateau values at 0.97 ± 0.04 l/hr/kg (well-stirred, Figure 3) and 1.11 ± 0.05 l/hr/kg (parallel tube, Figure 3D). Adult clearances were reached at 1.3 months (well-stirred) and 1.0 months (parallel tube), respectively.
Figure 5.2. Protein expression of UGT1A9 in neonatal, pediatric, adolescent, and adult livers and correlation of protein to activity levels.
A: Representative western blots of UGT1A9. B: Protein expression increased with age, peaking at 10.0 months. Range for all samples was 0.78 – 155 density units and range of adult (>20 years) was 12.8 – 155 density units. C: Protein activity showed correlation to activity levels only less than 1 year of age. Two-tailed Spearman correlation returned P = 0.025 and r = 0.68. Band intensities were calculated using Image-J software (NIH) and were normalized to the pooled samples on each western blot. The dashed line represents the pooled sample (n = 200) while the dotted line is the averaged individual adult samples (n = 37).
Figure 5.3. The scaled hepatic clearance of 4MU by UGT1A9 in pediatric liver. 
A: Allometric PK model well stirred. Initial clearance starts at 2.05 and drops to a plateau to an apparent adult clearance of $1.12 \pm 0.07$ L/hr/kg at 18.1 years. B: Allometric PK model, parallel tube. Initial clearance starts at 2.31 and drops to a plateau to an apparent adult clearance of $1.23 \pm 0.071$ L/hr/kg at 18.4 years. C: Simcyp PBPK model, well stirred. Initial clearance starts at 0.56 and plateau is reached at $0.97 \pm 0.04$ L/hr/kg at 1.3 months. D: Simcyp PBPK model, parallel tube. Initial clearance starts at 0.80 and plateau is reached at $1.11 \pm 0.05$ L/hr/kg at 1.0 months. All models are fit from ages 0 to 20 years, un-weighted, and constrained by medium convergence criteria requiring five consecutive iterations of the fit to change the sum-of-squares by less than 0.0001%. The dashed line represents the pooled sample ($n = 200$) while the dotted line is the averaged individual adult samples ($n = 45$).
Table 5.2. Summary table for modeled 4MU clearance values.

<table>
<thead>
<tr>
<th>UGT1A9 Clearance Parameter</th>
<th>Well-Stirred</th>
<th>Parallel Tube</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Allometric</td>
<td>0.9713</td>
</tr>
<tr>
<td></td>
<td>Simcyp</td>
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<tr>
<td>Normalized Adult Clearance</td>
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</tr>
<tr>
<td>R²</td>
<td>0.6909</td>
<td>0.01026</td>
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<tr>
<td>95% CI of Adult Clearance</td>
<td>0.9736, 1.260</td>
<td>0.8924, 1.050</td>
</tr>
<tr>
<td>Age at Adult Activity</td>
<td>18.1 years</td>
<td>1.3 months</td>
</tr>
<tr>
<td>Absolute Sum of Squares</td>
<td>5.016</td>
<td>28.79</td>
</tr>
<tr>
<td>Sy.x</td>
<td>0.1935</td>
<td>0.4535</td>
</tr>
</tbody>
</table>

Clearances are expressed as L/hr/kg.

**DISCUSSION**

The development of UGT1A9 is age-dependent, reflecting similar published studies for UGT1A4 (Miyagi and Collier, 2007), 1A1 and 1A6 (Miyagi and Collier, 2011), and 2B7 (Zaya et al., 2006). The fitted model indicates that UGT1A9 activity is not present at birth and reaches “adult” (90% of maximum-derived levels) levels by 4 months of age which is in good agreement with the finding of Strassburg et al. (2002).

Previously, we postulated that isoforms of the UGT1A subfamily are post-transcriptionally modified and/or demonstrate allosteric mechanisms responsible for activity modulation because protein expression did not correlate to activity for UGT1A1.
or 1A6 (Miyagi and Collier, 2011). Herein, UGT1A9 positively correlates protein expression with activity as the enzyme develops in the neonate. This finding is similar for UGT2B7 (Zaya et al., 2006). Interestingly, in adults the correlation between protein expression and activity is lost. There are several possible explanations. The antibody used in this study and the ECL detection system (compared with TMB previously) may have allowed for more sensitive western probing. To test this, we re-probed the blots using an antibody against different UGT1A6. Even with more sensitive methods, expression of UGT1A6 protein was not age-dependent and did not correlate with enzyme activity (data not shown). Therefore, we speculate that during neonatal development, hepatic UGT1A9 expression is driven by genetic (transcriptional) production. Evidence seems to indicate that once maximal activity is reached, post-transcriptional and/or allosteric mechanisms modulate 1A9 activities, providing a level of environmental responsiveness. To better understand this phenomenon, further examination of the molecular mechanisms driving hepatic UGT1A9 expression is necessary.

Some characteristics of the molecular regulation of UGT1A9 are known. Barbier et al. (2003) demonstrated enhanced expression and activity in hepatocytes with the treatment of peroxisome proliferator-activated receptor α and γ activators. Additionally, UGT1A9 is regulated by hepatocyte nuclear factors (HNF) 1α and 4α, with binding of HNF4α being essential for up-regulation (Gardner-Stephen and Mackenzie, 2007). More recently, aryl hydrocarbon receptor signaling and nuclear factor erythroid-related factor 2 binding to antioxidant and xenobiotic response elements have been implicated in transcriptional regulation of UGT1A9 (Kalthoff et al., 2010). These studies support our speculation that
development of UGT1A9 is transcriptional in the neonate, but that the loss of protein/activity concordance in children over 1 year old is due to genetic and environmental influences including nuclear receptor cross-talk.

An interesting and novel finding from this study is the effect of sub-cellular fractionation and freeze-thaw cycles on apparent UGT protein expression by western blot. Eight individual liver samples were originally received as S9 fractions. The frozen S9 was thawed, microsomes were prepared by differential centrifugation then aliquoted and re-frozen before a third thaw for activity assays. Although activities were not markedly different between these eight samples and the other individual samples, western blots uniformly showed approximately 11-fold higher levels of protein. Since activities between the in-house S9/microsomal preparations and commercially-processed microsomes were the same, this finding has important implications. When attempting to pool samples from different laboratories, consistent sample preparation and storage parameters will be vital to prevent confounding of protein expression results.

There was also divergence between the allometric PK and Simcyp PBPK model for the maturation of 4MU clearance by UGT1A9. The allometric PK model returned high initial clearances that decreased to adult levels over many years, with neonatal clearance being almost twice the adult clearances. In contrast, Simcyp PBPK returned low neonatal clearances that increased to a plateau. In the Simcyp PBPK scenario, neonatal clearance values for 4MU were half of adults. These differences may be attributed to individual factors included in the PBPK model that the PK model does not take into account.
Alternatively, since only enzyme activity, albumin, \( Q_{\text{hepatic}} \), and MPPGL maturation are directly used in the PBPK model, the allometric PK model (scaled to \( \frac{3}{4} \) using only body weight) may be less precise, but more robust to other maturation factors that have not been specifically included. Additionally, Anderson and Holford (2009) compared scaling with BSA (our PBPK model) to the \( \frac{3}{4} \)-allometric scaling (our PK model) and concluded that the \( \frac{3}{4} \)-model is generally better. Therefore the error in the BSA scalar may have propagated itself when used to calculate whole liver clearance, giving an underestimated value. We have stated previously the importance of assessing which models may be superior for certain data types. It seems that for UGT1A9/4MU clearance, the allometric model is more accurate, and this may be an important characteristic when considering scaling for high extraction drugs.

Of note is the similarity between our derived allometric 4MU/UGT1A9 clearance and reported clinical clearance of propofol in children. The 4MU/UGT1A9 clearance in this study was flow limited which is similar to clinical propofol clearance, an exclusive substrate for UGT1A9 (Murat et al., 1996; Rowland et al., 2008). Children need higher propofol dosages since clearance is greater than in adults. Past speculation has included that pediatric dosing differences for propofol were due to PK and/or drug sensitivity (Peeters et al., 2006) and the data presented seem to indicate that higher propofol clearance in children is pharmacokinetic. The average propofol clearance in children under 3 years of age from four previous studies is \( 3.2 \pm 1.3 \) l/hr/kg (Vandermeersch et al., 1989; Murat et al., 1996; Rigby-Jones et al., 2002; Peeters et al., 2006). These values are similar to our modeled hepatic clearances of 4MU (a drug with similar flow-limited
clearance) of 2.1 and 2.3 l/hr/kg (well-stirred and parallel tube models, respectively).

Adult propofol clearance has been estimated at 1.6 l/hr/kg, which is approximately 30% greater than normal hepatic blood flow due to extra-hepatic propofol clearance (Yamashita et al., 2010). Although we have not determined the contribution of non-hepatic UGT1A9 metabolism (i.e. the kidney and intestine), if the data presented for pediatric clearance of 4MU are increased by 30% (to estimate the effect of extra-hepatic clearance as shown by Yamashita et al.), they reach approximately 2.8 l/hr/kg, which is very similar to the average pediatric propofol clearance. While the $K_m$ and $V_{\text{max}}$ for propofol differ from 4MU (41 $\mu$M and 2.3 nmol/min/mg protein versus 8.0 $\mu$M and 28 nmol/min/mg protein), the intrinsic clearances are approximately the same (3.3 versus 2.5 ml/min/mg protein for propofol and 4MU, respectively) (Rowland et al., 2008). Since the intrinsic clearances are similar, elimination of both drugs will be limited by hepatic blood flow. Seemingly, UGT1A9 has high capacity for xenobiotics leading to high extraction. This fact may explain why other UGT1A9-metabolized drugs, including SN-38, and mycophenolic acid, require greater dosages in children than in adults (Thompson et al., 2008; Ettenger et al., 2005).

The data presented concur with the findings of Anderson and Holford (2009) by showing that the allometric $\frac{3}{4}$-scalar was more accurate than using BSA, especially for high extraction drugs where clearance is flow-limited. Comparisons between PK and PBPK models and evaluation of model-specific parameters are vital to strengthening the sensitivity and robustness of *in silico* approaches for pharmacokinetic analyses. It seems that for the UGTs, differing models return similar predictions of clearance and maturation...
for low extraction chemicals where the critical pathways are not flow-limited (Miyagi and Collier, 2007; Miyagi and Collier, 2011). However, this is not the case for UGT1A9 where the allometric model predicts maturation at age 18 while the Simcyp PBPK model matured at 1 month of age. One potential reason is that compared to the cytochromes P450 (P450), scaling of UGTs is under-predictive (Miners et al., 2006; Miyagi and Collier, 2011). The latency of UGTs has been reasoned as a cause since CYPs do not have this characteristic (Radominska-Pandya et al., 1999). We postulate another reason which stems from the original method used to derive the standard MPPGL (45 mg/g liver tissue). Hakooz et al. (2006) used total protein determination along with the P450 spectrum shift and cytochrome C reduction by P450 reductase to generate the relationship of MPPGL. This number is now used for scaling all liver microsomal content and has not been problematic since most research centers on drugs cleared by P450. However, for substrates that are not primarily cleared by hepatic P450s and do not have flow limited clearance, scaling may be confounded since the relative amount of other enzymes in microsomes may differ from P450 content. In future, improving scaling for drugs metabolized through pathways distinct from P450s may involve a different scalar for MPPGL that is specific to the enzyme family of interest.

The most significant finding of this study is in the description of UGT1A9 maturation in the neonate where protein expression and activities correlate. Thereafter, for UGT1A9 we did not observe a protein:activity relationship in the child or adult population, likely demonstrating the interplay of genetic and environmental variables. These data imply that genetic, more so than environmental mechanisms, are responsible for activating UGT1A9
in the neonate, but in children and adults; UGT1A9 activity is modulated by additional mechanisms. Ultimately, through understanding the attributes unique to pediatric biotransformation, we will be able to reduce adverse drug and chemical reactions in children and better serve the pediatric medical community.
REFERENCES


CHAPTER 6

GENERAL DISCUSSION
DISCUSSION

The research presented in this dissertation provides, for the first time, a description of the functional ontogeny of both hepatic antioxidants and hepatic UGT1A detoxification enzymes. Results demonstrate highest hepatic antioxidant capacity in the neonate that declines throughout childhood to a nadir around the age of ten then increases to adult levels after adolescence. For UGTs the general pattern in activity is negligible or low at birth and then increase to a plateau after a number of months. Clearance matures later taking one to five years.

Additionally, the hepatic development of the major endo- and xenobiotic metabolizing UDP-glucuronosyltransferase 1A isoforms, namely UGT1A1, UGT1A4, UGT1A6, and UGT1A9 isoforms was determined. Each of the UGT1A isoforms are independently regulated and mature at different rates, although enzymes generally showed adult levels of activity by two years of age. Of note, UGT1A1, UGT1A4 and UGT1A6 maturation is likely to be regulated at the post-translational level by direct modification of the enzyme protein and/or the effects of allosteric interactions on enzyme action. In contrast, UGT1A9 appears to mature primarily through transcriptional (genetic) effects, up to one year of age. When UGT1A9 reaches adult levels, the direct relationship between protein and activity observed in neonates is lost, indicating the effects of environmental variables and the high levels of environmental responsiveness in UGT1A9. For all isoforms, hepatic clearance matured after maximum enzyme activity, ranging from one to six years of age indicating that physiological maturation of the liver was important as well. Lack of detoxification through the UGT pathway, (arguably the most important Phase II system)
in the neonatal period and early childhood coupled with the antioxidant nadir discovered by us has implications for chemical, drug and endogenous toxicity. Moreover, due to the critical role of both UGTs and antioxidants in mediating homeostasis, we believe that developmental lack of these pathways in childhood plays a role in pediatric liver diseases, including non-alcoholic fatty liver disease (NAFLD). Finally, the studies presented in this dissertation have played a role in the development and refinement of pediatric pharmacokinetic models, thereby improving in silico approaches to model pediatric drug disposition. Overall, data presented herein have added substantially to the fields of developmental pharmacology and pharmacokinetics.

When considering pediatric liver toxicity and the development of liver disease, the “two-hit” hypothesis is the currently accepted theory (Reddy and Rao, 2006). The process of NAFLD begins with hepatic lipid accumulation that causes increases in reactive oxygen species (ROS). The over-abundance of ROS leads to oxidative stress, inflammation, and over prolonged periods of time, eventual fibrosis and end-stage NAFLD, non-alcoholic steatohepatitis (NASH). Recent experiments show palmitic acid, a dietary free fatty acid (FFA), can up-regulate the inflammasome (Csak et al., 2011), triggering activation of surrounding immune cells and starting the process of inflammation. Prolonged inflammation can lead to liver damage and cirrhosis as observed in chronic cases of Hepatitis C (Simula and De Re, 2010). Similar to Hepatitis C, not all cases of pediatric NAFLD lead to NASH and liver cirrhosis (Caruntu and Benea, 2006). Herein, we propose an updated model to the “two-hit” hypothesis. Errors in antioxidant and detoxification capacity are risk factors for pediatric NAFLD; lower activity levels of
Antioxidant and detoxification may allow excessive ROS to accumulate in the liver, particularly by bile acids, and may provide the impetus for the start of pediatric NAFLD (Figure 6.1).

**Figure 6.1. Updated model of the “two-hit” hypothesis.**
Fatty liver and NAFLD are highly correlated with insulin resistance and obesity. Diet, drugs and toxins can also contribute to NAFLD. We propose that developmental inadequacy (through environmental and/or genetic factors) of detoxification and metabolism can also contribute to NAFLD. The buildup of ROS, particularly from bile acids, due to reduced antioxidant and detoxification capacity will lead to excessive oxidative stress and eventual liver damage.

Antioxidants are enzymes and compounds that prevent cell damage by eliminating ROS. Inadequacy of this “antioxidant defense network” can ultimately lead to organ damage and systemic toxicities. This is seen in cases of acetaminophen-induced hepatotoxicity. In normal doses, acetaminophen is cleared from the body without any adverse
consequences. During detoxification, acetaminophen reactive metabolites are formed, but are detoxified by glutathione (GSH). However in overdose, GSH is depleted, allowing the reactive metabolites to covalently bond to proteins, which cause cell necrosis, increased oxidative stress, and liver damage (Hinson et al., 2004). Herein, the entire hepatic antioxidant defense network (with the exception of nitric oxide synthase) was profiled. We have shown that hepatic antioxidant capacity is highest in the neonate and declines throughout childhood to a nadir around the age of ten years. Subsequently, antioxidant capacity rebounds to adult levels by adolescence. While neonates and adults seem to be protected from excessive ROS through the antioxidant defense network, children seem to be more vulnerable due to a naturally lower antioxidant capacity.

The age where the antioxidant nadir appears to occur immediately precedes the usual age of clinical diagnosis of pediatric fatty liver disease. This convergence provides a plausible explanation for the restricted age profile of fatty liver disease in children, NAFLD. Furthermore, if these antioxidant compounds are depleted and/or the antioxidant enzymes are inhibited or have inherently low intrinsic activity, this would allow increased oxidative stress within the pediatric liver, leading to hepatic damage (Figure 6.2). Mitra et al. (1992) showed glutathione-S-transferase (GST) activity is inhibited in vitro by fatty acids and fatty acid esters. We speculate that the antioxidant nadir of childhood may predispose children to liver damage and liver disease when this nadir combines with other factors such as obesity, developmental lack of detoxification or genetically low detoxification capacity.
Figure 6.2. Etiology of pediatric NAFLD.
Antioxidant capacity starts high at birth, decreases to a nadir between the ages of six to ten years of age and rebounds to adult levels during adolescence (black line). Detoxification capacity increases from birth reaching adult capacity, anywhere from one to five years of age (green line). If detoxification ability is inhibited (red dotted line) around the same time as the nadir, the lowered antioxidant capacity may not be able to compensate for the increased ROS. This may explain the age which pediatric NAFLD is clinically diagnosed.

In terms of detoxification enzymes, the UGTs are an important superfamily with a wide capacity for biotransformation of xenobiotics and endogenous compounds. Clinical data shows children are more susceptible to drug and chemical toxicity. Although it is widely believed that developmental lack of detoxification enzymes are the main cause of poor pharmacological outcomes, this “fact” has not been based on evidence-based studies. For some metabolizing enzymes, developmental lack does not occur, such as for the sulfotransferases and cytochrome P450 (CYP) 3A7 that are higher in neonates than in
children (de Wildt et al., 1999; Richard et al., 2001). Data presented suggests that even after enzyme maturation peaks, liver function has not fully developed due to physiological development (such as hepatic blood flow, albumin levels, and liver size).

These studies also demonstrate that physiological development of the liver is rate-limiting for drug clearance and detoxification since enzyme activities peak before hepatic clearance. Until the child reaches physiological and anatomical hepatic maturity, he or she is more susceptible to liver damage from accumulation of drugs and toxic chemicals. Another important aspect is the wide variability of UGT activities within the population. For example, UGT1A9 activity varies almost 8-fold in the adult population, therefore; hepatic injury may occur in those individuals who have low intrinsic UGT activity. In particular we speculate that in children, when the complementary antioxidant defense network is compromised, redundancy in clearance pathways is decreased by developmental lack of UGTs. Obesity may further compound this effect since some UGTs are inhibited by FFAs (Tsoutsikos et al., 2004; Rowland et al., 2008). Taken together, obese children are at higher risk of adverse drug and chemical reactions. Furthermore, in the etiology of liver diseases, if the child already has low levels of UGT-mediated clearance, they would be more susceptible to oxidative stress-induced liver damage due to diminished hepatic detoxification capacity. At the same time as the obese child experiences an age-related nadir in antioxidant capacity, if that same child also has developmentally, genetically or environmentally low levels of UGT-mediated clearance, it is speculated that these two phenomena may combine to produce the impetus for initiating NAFLD.
Lastly, these studies help to improve pediatric pharmacokinetic (PK) and physiology-based pharmacokinetic (PBPK) modeling. Allometric modeling works relatively well in scaling for obese adults and children over the age of two (Anderson et al., 2000). However, scaling for neonates and infants tend to over-predict clearances. Herein, we show the differences between the different models (PK versus PBPK and allometric versus Simcyp, Table 6.1). In some cases, both models are in excellent agreement with each other (UGT1A4). Other cases, the models did not converge or the differences in clearance maturation between models were years apart (UGT1A9). This indicates that one model cannot be applied in all situations; one model may be more accurate than the other in certain cases. For UGT1A9, propofol clearance is higher in the neonate than in adults, which was reflected in the Simcyp (PBPK) models but not the allometric models. These studies underlie the importance of careful planning in picking models to predict clearance in infants and neonates.

**Table 6.1. Summary table for UGT-modeled clearances.**

<table>
<thead>
<tr>
<th>Isoform</th>
<th>Parameter</th>
<th>Well-Stirred</th>
<th>Parallel Tube</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Allometric</td>
<td>Simcyp</td>
</tr>
<tr>
<td>UGT1A1</td>
<td>Clearance</td>
<td>0.007</td>
<td>0.0079</td>
</tr>
<tr>
<td></td>
<td>Age</td>
<td>3.0 years</td>
<td>2.6 years</td>
</tr>
<tr>
<td>UGT1A4</td>
<td>Clearance</td>
<td>0.005</td>
<td>0.005</td>
</tr>
<tr>
<td></td>
<td>Age</td>
<td>5.1 years</td>
<td>5.1 years</td>
</tr>
<tr>
<td>UGT1A6</td>
<td>Clearance</td>
<td>Did not</td>
<td>0.0079</td>
</tr>
<tr>
<td></td>
<td>Age</td>
<td>converge</td>
<td>12.6 months</td>
</tr>
<tr>
<td>UGT1A9</td>
<td>Clearance</td>
<td>1.117</td>
<td>0.9713</td>
</tr>
<tr>
<td></td>
<td>Age</td>
<td>18.1 years</td>
<td>1.3 months</td>
</tr>
</tbody>
</table>

Clearances are expressed as l/hr/kg.
FUTURE STUDIES – UGT INHIBITION BY FREE FATTY ACIDS

Currently, one in three Americans are clinically obese. These patients typically ingest numerous drugs to control medical problems arising from obesity such as hypertension, diabetes, cardiovascular disease, and renal dysfunction. Clinical data clearly demonstrate that obesity affects detoxification and elimination of drugs and chemicals (Boullata, 2010). However, these studies have generally focused on pharmacokinetic effects relating to absorption and distribution differences in obesity, with comparatively few efforts to determine effects of obesity and fat on enzymatic detoxification (metabolism and elimination). Research to date has generally focused on CYP effects (Buechler and Weiss, 2011), but there have been few studies examining associations and effects between Phase II enzyme activities and obesity. Preliminary data presented below suggests increasing obesity (as determined by body mass index, BMI) is associated with decreased hepatic detoxification.

Pooled liver samples of patients with BMIs of ideal weight, overweight, obese, and morbidly obese (n = 15, 10, 10, 5 for each pooled sample respectively) were used to evaluate the effects of obesity on hepatic detoxification. Interestingly, there was a significant decrease in UGT activity for obese and morbidly obese patients. The isoforms UGT1A1 (Figure 6.3) and UGT1A4 (Figure 6.4) activity showed significant decreases between those of ideal weight and morbidly obese patients. This may imply that BMI (or obesity) is associated with a liver-specific factor that inhibits detoxification enzyme activity.
Previous experiments show UGT activities are inhibited in vitro by FFAs (Rowland et al., 2008; Tsoutsikos et al., 2004). Fatty liver increases the amount of intracellular FFAs and the risk of fatty liver increases significantly with obesity (Dunn and Schwimmer, 2008). We speculate that the inhibition observed here with increasing BMI is due to FFAs effects. Increased FFAs may not only inhibit enzyme activity competitively (since they are substrates), but may also structurally alter enzyme proteins changing catalysis. The latter could explain the noncompetitive inhibition observed (Rouer et al., 1980).

Finally, UGT clearance shows dramatic decreases in morbidly obese individuals (Figures 6.1 and 6.2). This is a concern since UGTs metabolize many commonly prescribed drugs and are vital for nutrient and hormone balance. Taken together with data presented in previous sections of this dissertation demonstrating that normal clearance performed by antioxidants and UGTs does not reach adult levels till adolescence, early onset childhood obesity can predispose children to liver damage and liver disease. This is of great concern since both childhood obesity rates and pediatric fatty liver disease are already at epidemic proportions and still rising (Ogden et al., 2006).
Figure 6.3. Morbidly obese patients showed altered enzyme activity and clearance for UGT1A1.
A: UGT1A1 activity using 100 μM bilirubin. B: Normalized hepatic clearance for bilirubin. C: Western blot for hepatic UGT1A1. NW = Normal Weight (n = 15), OW = Overweight (n = 10), OB = Obese (n = 10), MO = Morbidly Obese (n = 5). Protein expression was calculated by area-density using ImageJ.
Figure 6.4. Morbidly obese patients showed altered enzyme activity and clearance for UGT1A4.
A: UGT1A4 activity using 100 μM trifluoperazine. B: Normalized hepatic clearance for trifluoperazine. C: Western blot for hepatic UGT1A4. NW = Normal Weight (n = 15), OW = Overweight (n = 10), OB = Obese (n = 10), MO = Morbidly Obese (n = 5). Protein expression was calculated by area-density using ImageJ.
**FUTURE STUDIES – MECHANISMS FOR FAT-INDUCED CHANGES**

While current NAFLD models involve the use of drugs or dietary supplements in mice, there have been few studies that assess the molecular pathogenesis of the disease. Although the two-hit hypothesis has been widely been accepted, it is unclear how fat accumulates in the hepatocytes and why the increased accumulations cause an increase in ROS. Using a human hepatocellular *in vitro* model of steatosis (Gómez-Lechón et al., 2007), the effects of intracellular lipid accumulation were investigated. When a 2:1 ratio of 1 mM oleic/palmitic acid was added to the culture medium, our preliminary data shows a 200 percent increase in intracellular lipids (Figure 6.5). The increase in intracellular FFA also caused an increase in ROS for males, but not for females (Figure 6.6). In pediatric NAFLD, the majority of the cases are diagnosed in males; this may imply that males are more susceptible to hepatic oxidative stress. Finally, there was increased binding to PPARs when there are increased intracellular FFAs, with significant binding happening in males to PPARγ (Figure 6.7). Since PPARs are important in lipid metabolism and homeostasis, if PPAR function is disrupted or deficient, it could lead to an overabundance of intracellular FFAs. In conjunction with increased FFAs, there would also be increased ROS and thus leading to oxidative stress that would start the progression of NAFLD.
Figure 6.5. Increased intracellular fatty acids when overloading primary human hepatocytes incubated in different fatty acid ratios.

C = control (no fat loading), F/2:1 = hepatocytes loaded with 1 mM of 2:1 oleic:palmitic acid, F/0:3 = hepatocytes loaded with 1 mM of 0:3 oleic:palmitic acid. * P < 0.05, ** P < 0.01. n.d. = no data.
Figure 6.6. Increased intracellular levels of fatty acids cause increased intracellular ROS in male primary human hepatocytes. 
C = control (no fat loading), F/2:1 = hepatocytes loaded with 1 mM of 2:1 oleic:palmitic acid, F/0:3 = hepatocytes loaded with 1 mM of 0:3 oleic:palmitic acid. ROS = reactive oxygen species, DCF = dichlorofluorescein. n.d. = no data.
Figure 6.7. Increased intracellular fatty acids cause greater levels of PPARγ binding activity in nuclear extracts in male primary human hepatocytes. C = control (no fat loading), F/2:1 = hepatocytes loaded with 1 mM of 2:1 oleic:palmitic acid, F/0:3 = hepatocytes loaded with 1 mM of 0:3 oleic:palmitic acid. PPAR = peroxisomal proliferation activator receptors. * P < 0.05, ** P < 0.01. n.d. = no data.
CONCLUSION

In summary, the work contained in this dissertation was the first to demonstrate the developmental dynamics of the antioxidant network and the major UGT1A isoforms in pediatric liver. Antioxidant status is high at birth and declines to a nadir in the age range of 6 to 10 years and rebounds to adult levels during adolescence. In contrast, UGT enzymes start low and increase to adult activities in childhood within a few months to a couple of years. Clearances mediated by UGTs mature much later, from one to 18 years of age. Differences in clearance can be attributed to the different models; models must be appropriately used to predict accurate clearance. Inhibition or dysregulation of the antioxidant or detoxification enzymes, through environmental or genetic factors, may predispose the child to higher risks of ROS due to the lowered ability to detoxify the liver.

Preliminary data also suggest that in addition to developmental mechanisms, drug detoxification can be diminished as FFA levels in the hepatocyte increase. These data confirm and support the “two-hit hypothesis” and further, provide critical junctures on which to focus. Namely, by elucidating the relationship between FFA, ROS and drug metabolism, including the interaction of FFAs, bile acids, antioxidant enzymes, and UGTs in hepatocytes, we can design strategies to mitigate or prevent pediatric adverse drug reactions in normal weight and obese children. Furthermore, these studies provide intriguing insight into the as-yet unknown mechanisms of pediatric NAFLD that bear further study.
REFERENCES


