Pediatric Cytochrome P450 Activity Alterations in Nonalcoholic Steatohepatitis

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Abbreviations: ADME, absorption, distribution, metabolism, and excretion; AUC, area under curve; BMI, body mass index; CYP, cytochrome p450; IS, internal standard; NAFLD, nonalcoholic fatty liver disease; NAS, NAFLD Activity Score; NASH, non-alcoholic steatohepatitis; and VDR, variable drug responses.

Abstract

Variable drug responses(VDRs) are dependent upon individual variation in the activity of drug-metabolizing enzymes including cytochrome P450s(CYPs). As the most common chronic liver disease in children and adults, nonalcoholic steatohepatitis(NASH) has been identified as a source of significant inter-individual variation in hepatic drug metabolism. Compared to adults, children present age-related differences in pharmacokinetics and pharmacodynamics. The purpose of this study was to determine the impact of fatty liver disease severity on the activity of a variety of CYPs in children and adolescents. Healthy and nonalcoholic fatty liver disease(NAFLD) pediatric subjects ages 12-21 years inclusive received an oral cocktail of four probe drugs: caffeine(CYP1A2,100mg), omeprazole(CYP2C19,20mg), losartan(CYP2C9,25mg), and midazolam(CYP3A4,2mg). Venous blood and urine were collected pre-administration and 1, 2, 4 and 6h post-administration. Concentrations of parent drugs and CYP-specific metabolites were quantified in plasma and urine using LC-MS/MS. In plasma, decreased metabolic area under the curve(AUC) ratio, defined as metabolite AUC to parent AUC, of omeprazole indicated significant decreases of CYP2C19(p=0.002) enzymatic activities in NASH adolescents, while the urine analyses didn't show significant differences and were highly variable. A comparison between the present *in vivo* pediatric studies and a previous *ex vivo* study in adult indicates distinct differences in the activities of CYP1A2 and CYP2C9. These data demonstrate that pediatric NASH presents an altered pattern of CYP activity and NASH should be considered as a confounder of drug metabolism for certain CYPs. These differences could lead to future investigations that may reveal unexpected VDRs that should be considered in pediatric dosage recommendations.

Introduction

Nonalcoholic fatty liver disease(NAFLD) encompasses a spectrum of liver disorders ranging from simple steatosis to the progressive form of non-alcoholic steatohepatitis(NASH), which is characterized by inflammation and hepatocyte injury that may lead to liver cirrhosis and even hepatocellular carcinoma(HCC)(Matteoni *et al.*, 1999; Chalasani *et al.*, 2012). Many studies demonstrate the entire spectrum of NAFLD also occurs during childhood(P. Molleston, 2002; Kleiner and Makhlouf, 2016). Concurrent with childhood obesity, NAFLD has developed into a serious concern in pediatric clinical practice, and become a significant health burden in adolescence(AlKhater, 2015). An autopsy-based study estimated the overall prevalence of NAFLD in the American pediatric population at 9.6%, which increased to 17.3% in adolescents from 15 to 19 years old(Schwimmer *et al.*, 2006).

In addition to the direct health concerns from NAFLD or NASH, potential adverse drug reactions(ADRs) associated with this underlying disease have received more and more attention in recent years. NASH has been identified as a potential risk factor for ADRs since profound reprogramming of drug metabolizing enzyme and drug transporter profiles were observed in NASH compared to healthy or simple steatosis populations(Fisher *et al.*, 2009; Hardwick *et al.*, 2011, 2013; Lake *et al.*, 2011). Additionally, in preclinical NASH models, pharmacokinetic alterations were observed for a number of drugs, including ezetimibe, pravastatin, methotrexate, morphine and metformin. (Hardwick *et al.*, 2012, 2014, Clarke *et al.*, 2014, 2015; Dzierlenga *et al.*, 2015). Each of these studies was conducted in adults or animal models reflecting the mature population, which underscores the scarcity of data on the impact of NASH on drug metabolism and disposition in children.

One study by Barshop et al. reported that following acetaminophen administration, children with NAFLD had significant higher serum and urinary concentrations of acetaminophen-glucuronide(APAP-gluc), indicating an altered drug metabolism and disposition process along with NAFLD in children(Barshop *et al.*, 2011). Canet et al. further demonstrated that regulation of hepatic efflux transporters is altered following the progression to NASH in children, which represents an underlying mechanism for increased APAP-gluc in children with NAFLD(Canet *et al.*, 2015).

Furthermore, compared to adults, children experience less certainty regarding drug safety. Physiological and ontological differences between children and adults result in age-related differences in pharmacokinetics and pharmacodynamics(Anderson *et al.*, 2010). Therefore, adult data regarding pharmacokinetics and pharmacodynamics cannot simply be extrapolated to children(Kearns *et al.*, 2003). Especially for critically ill children, who are usually exposed to multiple medications, pathological conditions introduce additional influence on drug processing(Vet *et al.*, 2011). A systematic review reported 42%-100% of pediatric patients received at least one off-label and/or unlicensed drug(Magalhães *et al.*, 2015), which is associated with high occurrence of ADRs(Mason *et al.*, 2012).

As the most important drug metabolizing enzyme superfamily, cytochrome p450s(CYPs) are a major source of variability in drug metabolism. Among 57 putatively functional human CYPs, CYP1A2, CYP2C9, CYP2C19 and CYP3A4 are relatively abundant, and responsible for the biotransformation of approximately 60% of all clinical drugs(Zanger and Schwab, 2013). Expression and enzymatic activities of the human CYPs are influenced by multiple factors such as genetic polymorphisms, age, gender, environmental toxicant exposure, dietary constituents, concomitant drug utilization, disease states and

others(Zanger and Schwab, 2013). Previously, we have reported a dramatic alteration of the CYP activity and expression profile in adult NASH patients in an *ex vivo* study, indicating that disease states such as NASH can alter drug pharmacokinetics by modulating CYPs(Fisher *et al.*, 2009). However, data regarding CYP activity in children with NAFLD are absent.

An accurate characterization of CYP activity is highly practical in predicting the fate of certain classes of drugs in a particular patient or a patient population that can guide dosing. Compared to an *ex vivo* study using biopsies, an *in vivo* approach which was applied in this study can yield greater accuracy since it is performed in the intact physiological context with dynamic blood flow and functioning liver.(Hisaka *et al.*, 2010) The purpose of the current study was to determine the *in vivo* activities of the major CYP isoforms along the spectrum of NAFLD severity in children and adolescents using isoform-specific probe drugs.

Patients and Methods

Study design and population

Twelve pediatric NAFLD patients between the ages of 12-21 years inclusive were recruited at Columbia University Medical Center (New York, NY) and Cincinnati Children's Hospital Medical Center (Cincinnati, OH). A screening evaluation based on medical history, physical examination, biochemical screening, and appropriate genotype for one or more of the CYP enzymes under investigation were conducted with potential candidates. Medication history of each subject was examined by screening their medical records and subjects under long-term or extensive medications that can potentially interfere with CYP

activities were excluded from the study. The NAFLD Activity Score (NAS) of each subject was given based on certified pathologists' evaluation of a percutaneous liver biopsy performed previously on each subject for diagnostic Standard of Care reasons. Briefly, pediatric patients without known allergies to study drugs and with biopsy-confirmed NAFLD with biopsy performed within 6 months of this study were eligible for inclusion. Seventeen healthy control subjects age 18 to 21 without any present or past critical liver diseases were recruited at University of Arizona Medical Center (Tucson, AZ). Subjects with BMI<30kg/m^2 and alanine aminotransferase (ALT) no higher than 26U/L for males, 22U/L for females were eligible for inclusion in the control group. Subjects with diabetes mellitus, monogenic inborn errors of metabolism, viral hepatitis, alcohol use, pregnancy, cirrhosis, or other causes of chronic liver disease were excluded from either disease or control group. This study and all study procedures were reviewed and approved by the University of Arizona, Columbia University Medical Center, and Cincinnati Children's Institutional Review Boards before study commencement. Parents or subjects who met all eligibility criteria gave written informed consent and informed assent after being given a description of the study before participation in the study.

Saliva was collected from participating subjects. DNA was isolated from the samples and genetic analysis of major relevant variants was performed for polymorphisms in CYPs.

Subjects were asked to abstain from caffeine containing food and drinks, charbroiled foods, cruciferous vegetables, grapefruit and grapefruit juice and any medications may influence CYP activities for 48 hours prior to and during participation in the study. After an overnight fast, a venous blood sample and a urine sample from each subject were collected immediately prior to drug administration. Subjects then received the probe drug cocktail

orally (100mg caffeine, 20mg omeprazole, 25mg losartan, and 2mg midazolam syrup), and post-administration blood and urine collections were obtained at 1, 2, 4, and 6-hour time points. Food and drink were allowed following administration, with the exception of prohibited foods and drinks listed above. Blood samples were centrifuged for plasma collection and aliquots were stored at -80°C.

Chemicals and Reagents

Caffeine citrate 198mg capsule, losartan potassium 25mg tablet, omeprazole 20mg capsule and midazolam HCl 2mg/ml were purchased through Reed's Compounding Pharmacy (Tucson, AZ).

Caffeine, losartan, omeprazole, midazolam, 1'-hydroxymidazolam and phenacetin standards for LC-MS/MS analysis were purchased from Sigma-Aldrich (St Louis, MO). Paraxanthine, paraxanthine-D3, losartan carboxylic acid (E3174) and 5-hydroxyomeprazole were purchased from Toronto Research Chemicals (Toronto, Ontario Canada). All other chemicals were obtained from commercial sources.

Cytochrome P450 genotyping

CYP2C9*2 (restriction enzyme analysis with AvaII on a 241 bp PCR fragment flanking the SNP), CYP2C9*3 (Sanger sequencing of a 259 bp. fragment flanking the SNP), CYP2C19*2 (restriction enzyme analysis with BstNI of a 201 bp. fragment flanking the SNP), CYP2C19*17 (Sanger sequencing of a 201 bp. fragment flanking the SNP), CYP3A4*1b (Sanger sequencing of a 216 bp. fragment flanking the SNP), and CYP3A5*3 (Sanger sequencing of a 182 bp. fragment flanking the SNP) typings were performed on all subjects. CYP3A5*6 (restriction enzyme digestion with DdeI of a 127 bp. fragment flanking the SNP) was performed on all Hispanics, CYP3A5*7 (Sanger sequencing of a

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227 bp. fragment flanking the SNP) was performed on all Afro-americans, and CYP2C19*3 (restriction enzyme analysis with BamHI of a 224 bp. fragment flanking the SNP) were performed on all Asian or Pacific Islanders. Cytochrome P450 genotyping primer sequences for the PCR fragment amplifications are available on request.

Laboratory analysis

According to the manufacture's protocol, an alanine transaminase (ALT) assay kit was used to perform the ALT quantification in the baseline plasma samples (Cayman Chemical, Ann Arbor, MI). A colorimetric creatinine assay was used to quantify the creatinine in the baseline urine of each subject (R&D system, Minneapolis, MN).

Sample preparation and LC-MS/MS method

Plasma samples (400μL) were spiked with an internal standard (IS, 10μL of 1000ng/mL Phenacetin), and were diluted by 1200μL HPLC grade water. 1600μL of the diluted sample was loaded into an EVOLUTE® ABN solid-phase extraction (SPE) cartridge (30mg, Biotage, Uppsala, Sweden), which was previously conditioned with 1 mL methanol and equilibrated with 1 mL water, and was washed with 1 mL 5% methanol twice and then eluted with 500μL methanol. The eluent was evaporated to dryness at room temperature under a stream of nitrogen, reconstituted in 100μL 40% acetonitrile, and a volume of 10μL was injected into the chromatographic system for separation and analysis.

The urine sample was 1:4 diluted with HPLC water, 400μL diluted urine was spiked with IS (10μL 1000ng/mL phenacetin and 10μL 4000ng/mL paraxanthine-d3). The spiked urine was processed with EVOLUTE® ABN SPE cartridge following the same procedure of plasma sample processing.

Analysis was performed with a Waters (Milford, MA) Micromass Quattro Premier XE tandem mass spectrometer coupled to an Acquity UPLC was used in the Arizona Laboratory for Emerging Contaminants at the University of Arizona. The analytes were separated using 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B) as mobile phases at a flow rate of 0.125 ml/min through a Waters Acquity UPLC BEH C18 column (1.7μm, 2.1×50mm). The UPLC gradient started at 40%B and increased to 50%B over 1.83min, sequentially increased to 100%B at 1.88min and continued for 0.9min, then was equilibrated to 40%B for 1 min before the next injection. Data acquisition was performed in multiple reactions monitoring mode with positive electrospray positive ionization. The mass transitions were as follows: $m/z195 \rightarrow 138$ for caffeine, $m/z181 \rightarrow 124$ for paraxanthine, $m/z346 \rightarrow 198$ omeprazole, $m/z362 \rightarrow 214$ for 5-hydroxyomeprazole, $m/z326 \rightarrow 291$ for midazolam, $m/z342 \rightarrow 324$ for 1-hydroxymidazolam, $m/z423 \rightarrow 207$ for losartan, $m/z437 \rightarrow 207$ for EXP-3174, $m/z180 \rightarrow 110$ for phenacetin, and $m/z184 \rightarrow 127$ for paraxanthine-D3.

Variance comparisons & statistical analyses

Data used for plotting pharmacokinetic curves are represented as mean ± standard error (SEM). Pharmacokinetics area under the curve (AUC) of parent drug or metabolite was computed for each subject. AUC ratio, defined as metabolite AUC divided by parent AUC, was computed and the box and whisker plots of AUC ratio represent the median and the min and max. Comparisons among normal, steatosis and NASH groups was performed with one-way ANOVA Bonferroni's multiple comparison test, and "*" indicates significance ($p < 0.05$) from control. All analyses were carried out using GraphPad Prism software (GraphPad Software, Inc., La Jolla, CA).

Enzymatic activity data collected from this study were compared with results from a previous *ex vivo* study conducted with adult NASH patients(Fisher *et al.*, 2009). Relative CYP enzymatic activity of each NASH subject was computed by normalizing to mean CYP activity of the normal group. For comparison, mean difference of CYP activity from steatosis or NASH group to control were computed from a linear model with logarithmic transformed relative activity.

Results

Clinical Characteristics of the Study Population

Clinical characteristics of study populations are summarized in Table 1. Seventeen healthy subjects were categorized as normal. According to the steatohepatitis diagnosis by liver biopsy, seven subjects were categorized as steatosis, while five were confirmed as NASH patients. The mean age and body mass index (BMI) were 19.3±0.3 years and 23.8±1.2 kg/m^2 for healthy population, 15.3±0.9 years and 33.1±2.0 kg/m^2 for steatosis and 17.4±0.7 years and 38.3±1.5 kg/m^2 for NASH. 47.1% of control, 71.4% of steatosis and 40% of NASH population were female. The control group had a diverse ethnic background, while 100% of steatosis and NASH subjects had Hispanic ethnicity. Compared to control group (17.7±1.5 U/L), NAFLD subjects had a significantly higher ALT level (79.3±26.3 for steatosis and 72.5±16.5 for NASH). The average NAS was 4.0±0.5 for steatosis and 5.6±0.2 for NASH. All NAFLD (steatosis and NASH) subjects showed significant steatosis with an average score of 2, indicating 34-66% fatty infiltration of hepatocytes with macrovesicular steatosis evident. NASH subjects had significant higher scores of lobular inflammation and hepatocellular ballooning than simple steatosis. Polymorphisms of CYP2C9*2, CYP2C9*3, CYP2C19*2, CYP2C19*17, CYP3A4*1b and CYP3A5*3 were

characterized in all subjects in this study, while CYP3A5*6 was performed on all Hispanics, CYP3A5*7 was performed on all Afro-Americans, and CYP2C19*3 was performed on all Asian or Pacific islanders. The numbers of polymorphic subjects of each CYP isoform are reported in Table 1.

Pharmacokinetics of probe drugs in plasma

The pharmacokinetic curves of probe drugs (caffeine, losartan, omeprazole and midazolam) and their metabolites (paraxanthine, EXP3174, 5-hydroxyomeprazole and 1-hydroxymidazolam) in plasma are shown in Figure 1A. The metabolic AUC ratio of each probe drug was used to represent the enzymatic activity of respective CYP isoform. The metabolic AUC ratio of omeprazole was decreased by 71% in NASH patients compared to healthy subjects, indicating a significant loss of CYP2C19 function in NASH adolescents, although there was no significant difference between steatosis and NASH groups. Even though the pharmacokinetic curves of caffeine, losartan and midazolam as well as their metabolites were differentiated among normal, steatosis and NASH groups, calculation of metabolic AUC ratios offset these changes, suggesting that enzymatic activity of CYP1A2, CYP2C9 and CYP3A4 were not significantly altered in adolescents with steatosis or NASH. Polymorphic subjects were excluded from the pharmacokinetic data analysis of the corresponding probe drug, although values of AUC ratio of polymorphic subjects are summarized in Tables 2, 3, and 4.

Pharmacokinetics of probe drugs in urine

Direct urinary concentration was normalized to urinary creatinine concentration (one healthy subject urine sample was absent, n=16). The pharmacokinetic curves were graphed

using normalized urinary concentrations (Figure 2A). Compared to plasma, urine data were more variable due to the inconsistency of urine samples. Significance for altered metabolic AUC ratios of probe drugs was not achieved in urine analysis, however, the decreasing trend in metabolic AUC ratio of omeprazole was consistent with plasma data and suggesting a decrease in CYP2C19 activity. As with the plasma analysis, polymorphic subjects were excluded from the data analysis, and values of polymorphic subjects are reported in Tables 2, 3, and 4.

Variance comparison between adolescent and adult studies

A variance comparison of CYP activities was performed between the present pediatric study and a previous study in adults(Fisher *et al.*, 2009). Relative enzymatic activities of steatosis and NASH subjects from both studies are presented in logarithmic scale in Figure 3. CYP1A2 activity was significantly decreased, and CYP2C9 activity was increased in adults with NASH, which was not shown in adolescents. In adults, CYP2C19 activity was significantly decreased in both steatosis and NASH, while a significant decrease of CYP2C19 activity was seen only in adolescents with NASH.

Discussion

Although pediatric NAFLD shares similarities with adult NAFLD with respect to a close association with obesity and cardiometabolic risk factors, there are known differences in some histological features, as well as environmental toxins and pubertal hormonal exposures, which may influence natural history and pathogenesis differently at younger ages(Crespo *et al.*, 2016). Therefore, adult data for drug metabolism and disposition alterations in NAFLD and NASH cannot simply be extrapolated to children. This study

attempted to characterize *in vivo* CYP activities in children with NAFLD, in order to precisely reflect the impact of NAFLD on CYP associated drug metabolism in the pediatric population.

Pediatric subjects are categorized by the FDA as adolescents between the ages of 12-21. The University of Arizona Institutional Review Board required that control subjects for this study be in the age range of 18-21 years. Age-related changes of CYPs have been extensively studied in this age range, and the most dramatic changes are observed in earlier stages of infanthood to early childhood(Wood *et al.*, 2003; Salem *et al.*, 2013). Additionally, since most of the current study subjects had reached puberty, the minor inequality of ages between groups is not likely to introduce significant variability in CYP activity.

The occurrence of NAFLD in children involves a complex interaction of genetic and environmental contributing factors(Loomba *et al.*, 2009). A strong association between NAFLD in children and childhood obesity is well established(Schwimmer *et al.*, 2003). In this study, a BMI of <30 kg/m2 was applied as an inclusion criteria for the control group, which excluded subjects with obesity from this group. As expected, patients with histologically-confirmed NAFLD had a significantly higher BMI. Ethnicity is another traditional risk factor of NASH with a high prevalence of NASH in patients with Hispanic origin(Bambha *et al.*, 2012), which was reflected in the ethnic composition of the NASH group in the study. A significant increase of alanine transaminase (ALT) was observed in steatosis (79.3±26.3 U/L) and NASH group (72.5±16.5 U/L) compared to control group (19.1±2.8 U/L), which is consistent with previously reported elevated ALT in pediatric NAFLD(Molleston *et al.*, 2014).

Heritable genetic variations in CYPs can significantly alter function of these enzymes. In order to minimize this variability, genotyping of high frequency single nucleotide polymorphisms (SNPs) were performed for all subjects. CYP2C9*2 and CYP2C9*3 are two most frequent and best investigated SNPs of CYP2C9, both of which lead to reduced CYP2C9 activity(Kidd *et al.*, 2001; Rettie and Jones, 2005). CYP2C19*2 is a high frequency allele in Caucasians, leading to reduced function of CYP2C19 and a decrease in the elimination of omeprazole(Zhou *et al.*, 1999) , while CYP2C19*17 is a gain-of-function allele leading to increased activity of CYP2C19(Zabalza *et al.*, 2012). CYP3A4*1b occurs in Caucasian population at 2~9%, though the effect on CYP3A4 activity is controversial(Zanger and Schwab, 2013). Polymorphic subjects with a specific SNP were excluded from the data analysis of the corresponding CYP isoform, though the AUC ratio values of these subjects are reported separately in Table 2-4. In addition, the majority of racial/ethnic differences in drug metabolism have been attributed to genetic polymorphisms(McGraw and Waller, 2012; Zanger and Schwab, 2013). Exclusion of polymorphic individuals in the current study design limits any potential racial/ethnic influence.

Alterations of metabolizing enzyme activities have been observed in critical diseases as well as compromised conditions in children. NAFLD is a complex disease that can be attributed to a variety of factors, including central obesity, diabetes mellitus, and dyslipidemia(Schwimmer *et al.*, 2003; Newton *et al.*, 2016). The specific impact of obesity on drug metabolism and elimination has been investigated intensively. To summarize these studies, clearance mediated by CYP1A2, CYP2C9, CYP2C19 and CYP2D6 tended to be higher in obese individuals, indicating an increase of metabolic activity of these CYPs,

although most studies were conducted *in vivo* in adults without statistically significance(Brill *et al.*, 2012). In contrast, in an *in vivo* evaluation of CYP1A2 activity in children with obesity between 6~10 years using urinary metabolism ratio of caffeine, a non-significant lower CYP1A2 enzymatic activity was reported(Chiney *et al.*, 2011). Additionally, in people with obesity, multiple known CYP3A4 substrates demonstrated decreased metabolism and clearance *in vivo*, with some achieving statistical significance, indicating a trend towards lower CYP3A4 metabolic activity in obese individuals(Brill *et al.*, 2012).

A critical component of NASH, inflammation has also been recognized as an important factor in altering drug metabolism. Reduced activity of CYP3A, CYP1A2, CYP2C9, CYP2C19 and CYP2D6 were reported in patients with an acute infection or inflammatory disease, and further mechanistic studies in animals and human hepatocytes showed that both lipopolysaccharide (LPS) exposure and the presence of elevated cytokines (IL-1, IL-6, TNF-α, IFN-γ and TGF-β) could lead to down regulation of CYP activity(Morgan, 1997; Aitken and Morgan, 2007; Yang *et al.*, 2010). In addition, inflammation has been shown to affect the expression and activity of important drug transporters(Cressman *et al.*, 2012). Interestingly, inflammation has been recognized as a major driving force in the progression from simple steatosis to steatohepatitis(Stojsavljević *et al.*, 2014). In a previous global gene expression study, the vast majority of altered absorption, distribution, metabolism, and excretion (ADME) genes were observed in NASH rather than simple steatosis, suggesting that ADME reprograming in NAFLD could be a simultaneous or sequential event of the second-hit in NASH progression associated with the inflammatory response(Lake *et al.*, 2011). In the current study, a significant decrease of CYP2C19 activity were observed in

adolescents with NASH but not steatosis, while no significant changes were found in CYP1A2, CYP2C9 and CYP3A4(Figure 1&2), which is partially consistent with data from other inflammatory conditions, but very different from observations in populations with obesity(Brill *et al.*, 2012). Although the significance was only observed in plasma samples but not urine samples, this may be due to variability in urine data obtained from the analysis. In adults, the impact of NASH on ADME pathways has been well-established(Fisher *et al.*, 2009; Hardwick *et al.*, 2010, 2011, 2013). In a previous *ex vivo* study in adult NASH, diverse patterns of CYP alterations were observed(Fisher *et al.*, 2009). For CYP1A2, a significant decrease in *ex vivo* activity occurred with a decreasing trend of mRNA expression as well as a significant decrease of protein expression. CYP2C9 activity was significantly increased, though neither mRNA nor protein expression was changed indicating a post-translational mechanism of regulation. However, changes in CYP1A2 and CYP2C9 activity were not found in either steatosis or NASH adolescents. A significant decrease in CYP2C19 activity was observed in both NASH adults and adolescents. Although there was some decrease in adult CYP3A4 activity without statistical significance, the CYP3A4 activity in adolescents was not significantly changed. In summary, the distinct pattern of CYP activity alterations in adolescents with NASH compared to adults suggests the possibility of ontogenetic factors in the response to inflammatory disease for these CYP isoforms. Although pharmacokinetic studies have demonstrated age-related differences in very young patients for some CYP activities, the present study demonstrates differences in response to disease in patients as old as 12 to 21 years, and age at which the activity of all CYP isoforms investigated were thought to be similar to adults(Hines, 2007). It is noteworthy that the *ex vivo* study was performed with

human liver microsomes, while the CYP activities in this pediatric study were evaluated in an intact physiological condition. Therefore, the differences found between the two studies may reflect the differences in CYP activities in the different reaction environments.

CYP-mediated drug metabolism in adolescents with NASH can decrease up to 80%, which creates the potential for an increased incidence of VDRs. Particularly for those drugs with severe side effects, a dramatic decrease of drug metabolism and elimination can have significant implications on the therapeutic use of a drug, and increase the risk of ADRs or drug toxicities. Furthermore, in addition to CYPs, alterations in other drug metabolizing enzymes and important drug transporters have been demonstrated in adult NASH patients(Hardwick *et al.*, 2010, 2011, 2013) as well as pediatric NASH patients(Canet, Merrell, Hardwick, Bataille, Campion, Ferreira, Xanthakos, Manautou, Hesham A-Kader, *et al.*, 2015). In other words, patients with progressive NASH may have a profoundly different context regarding drug biotransformation and disposition. This makes the prediction of pharmacokinetics of drugs in this population very difficult, and may have an implication on clinical drug trials. Though data of other ADME genes in pediatric patients with NASH is absent, additional differences between children and adults could be expected. Therefore, future research regarding the unique response of children to NASH, and in particular, pharmacokinetic studies of high-risk drugs are warranted in pediatric NASH patients. Physiologically based pharmacokinetic (PBPK) modeling may be particularly useful in translating the current findings into potential clinical implications and facilitating estimates for appropriate dose adjustments.

In conclusion, this study demonstrates a significant decrease in *in vivo* activity of CYP2C19 in adolescent NASH patients, whereas CYP1A2, CYP2C9 and CYP3A4 activity

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were unchanged, distinct from what was found in the *ex vivo* study in adults. These data indicate that adult and adolescents with NASH may need to be considered as unique patient populations in regards to CYP metabolism. Future studies on pharmacokinetics of high-risk substrate drugs in larger research populations will be needed to translate these findings to guide actual clinical practice. Ultimately, in pediatric clinical practice, in order to adequately characterize pharmacokinetics of CYP-metabolized drugs as well as make appropriate dose adjustment, the possibility of NASH should be taken into consideration.

Authorship contributions:

Participated in research design: Canet, Xanthakos, Lavine, Erickson and Cherrington.

Patient recruitment: Xanthakos, Lavine and Erickson.

Conducted experiments: Li, Clarke and Erickson.

Performed data analysis: Li and Billheimer.

Wrote or contributed to the writing of the manuscript: Li, Canet, Clarke, Xanthakos, Lavine, Erickson, and Cherrington.

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Footnotes

Figure Legends

Figure 1: Plasma concentrations and kinetics of probe drugs

The plasma concentrations of the probe drugs and their metabolites were measured, and the kinetics curves were graphed by plasma concentrations across 0, 1, 2, 4, and 6-hour time points, representing mean ± SD (A). Area under curve (AUC) was calculated for each probe drug and its metabolite. AUC ratio, defined as ratio of metabolite AUC to parent AUC, was calculated for each pair of probe drug and metabolite to represent in vivo activity of the corresponding CYP isoform (B). Box and whisker plots represent the median and the min and max, n=17 for control group, n=7 for steatosis group and n=5 for NASH group, subjects with CYP polymorphism were excluded from analysis, "*" indicates significant ($p<0.05$) from normal according to a One-way ANOVA Bonferroni's Multiple Comparison Test. $p=.5615$ for CYP1A2, $p=.201$ for CYP2C9, $p=.002$ for CYP2C19, while change in NASH group is significant from normal according to post test, and $p=.084$ for CYP3A4.

Figure 2: Urinary concentrations and kinetics of probe drugs

The urinary concentrations of the probe drugs and their metabolites were measured, and normalized to creatinine concentrations, and the kinetics curves were graphed by normalized urine concentration across 0, 1, 2, 4, and 6-hour time points, representing mean ± SD (A). AUC was calculated for each probe drug and its metabolite. AUC ratio, defined as ratio of metabolite AUC to parent AUC, was calculated for each pair of probe drug and metabolite to represent *in vivo* activity of the corresponding CYP isoform (B). Box and whisker plots represent the median and the min and max, n=16 for control group, n=7 for

steatosis group and n=5 for NASH group, subjects with CYP polymorphism were excluded from analysis, "*" indicates significant (p<0.05) from normal according to a One-way ANOVA Bonferroni's Multiple Comparison Test. $p=.8961$ for CYP1A2, $p=.8876$ for CYP2C9, $p=.4754$ for CYP2C19, and $p=.7214$ for CYP3A4.

Figure 3: Variance comparison of relative CYP activity

Adult data were extracted from a previous *ex vivo* study, which was performed with human liver microsomes, while adolescent data were generated in the current *in vivo* study within an intact physiological *context*. The relative enzymatic activities of NASH subjects were computed by normalizing to mean activity of control group. Data are presented in a logarithmic transformed scale. Each data point represents a measurement from one steatosis or NASH subject, while mean ± SEM is shown as well. "†" indicates significant (p<0.05) from normal within the group according to a One-way ANOVA Bonferroni's Multiple Comparison Test.

Table 1. Baseline characteristics of the study population

	Mean (SD)		
	Healthy	Steatosis	NASH
Age, y	19.3(1.2)	15.3(2.3)	17.4(1.7)
Female sex, No. (%)	8(47.1)	5(71.4)	2(40)
Hispanic ethnicity, No. (%)	3(17.6)	7(100)	5(100)
Race, No. (%)			
Asian or Pacific Islander	5(29.4)	0	0
Caucasian	10(58.8)	0	0
≥ 2 Races	0	1(14.3)	0
Height, cm	170.3(11.0)	163.5(10.2)	166.5(11.6)
Weight, kg	69.3(15.4)	89.6(24.4)	107.2(22.5)
BMI, kg/m^2	23.8(4.8)	33.1(5.3)	38.3(3.3)
ALT, U/L	18(6)	79(70)	73(37)
Liver histology			
NAFLD activity score (NAS)	N/A	4.0(1.4)	5.6(0.5)
Steatosis score	N/A	2.1(0.7)	2.2(0.8)
Lobular inflammation score	N/A	1.4(0.8)	2.0(0.0)
Portal inflammation score	N/A	0.6(0.5)	0.6(0.5)
Hepatocellular ballooning score	N/A	0.4(0.5)	1.6(0.9)
Fibrosis stage	N/A	1(1.7)	2(2.3)

CYP Polymorphisms No. (%)			
CYP2C9*2	4(23.5)	0	1(20)
CYP2C9*3	4(23.5)	1(14.3)	0
CYP2C19*2	4(23.5)	1(14.3)	0
CYP2C19*17	4(23.5)	0	1(20)
CYP3A4*1b	3(17.6)	3(42.9)	1(20)
CYP3A5*3	5(29.4)	4(57.1)	1(20)

Data are presented as mean (SD) or No. (%). BMI was calculated as weight in kilograms divided by height in meters squared. NAFLD activity score was assessed on a scale of 0–8, with higher scores showing more severe disease (the components of this measure were steatosis [assessed on a scale of 0–3], lobular inflammation [assessed on a scale of 0–3], and hepatocellular ballooning [assessed on a scale of 0–2]). Portal inflammation was assessed on a scale of 0–2, with higher scores showing more severe inflammation. Mean fibrosis stage was assessed on a scale of 0–4, with higher scores showing more severe fibrosis.

Table 2. Metabolic AUC ratio of Losartan in CYP2C9 polymorphic subjects

	Genotype	Plasma AUC ratio (SD, No.)	Urine AUC ratio (SD, No.)
Control	Reference Sequence(CC/AA)	2.45(1.73,9)	0.58(0.25,8)
	CYP2C9*2(CT/AA)	1.23(0.56,4)	0.51(0.21,4)
	CYP2C9*3(CC/AC)	1.53(0.84,4)	0.67(0.27,4)
Steatosis	Reference Sequence(CC/AA)	2.68(1.94,6)	0.53(0.11,6)
	CYP2C9*3(CC/AC)	0.19(N/A,1)	0.13(N/A,1)
NASH	Reference Sequence(CC/AA)	0.80(0.53,4)	0.54(0.14,4)
	CYP2C9*2(CT/AA)	2.06(N/A,1)	0.35(N/A,1)

Data are presented as mean (SD, No.).

Table 3. Metabolic AUC ratio of Omeprazole in CYP2C19 polymorphic subjects

	Genotype	Plasma AUC ratio (SD, No.)	Urine AUC ratio (SD, No.)
Control	Reference Sequence(GG/CC)	1.24(0.46,10)	51.00(55.19,10)
	CYP2C19*2(GA/CC)	0.63(0.20,3)	8.56(9.25,3)
	CYP2C19*17(GG/CT)	0.54(0.47,2)	43.54(38.39,2)
	CYP2C19*17(GG/TT)	0.87(N/A,1)	17.14(N/A,1)
	CYP2C19*2&CYP2C19*17(GA/CT)	1.64 (N/A,1)	26.18(N/A,1)
Steatosis	Reference Sequence(GG/CC)	0.55(0.51,5)	38.05(37.71,6)
	CYP2C19*2(GA/CC)	0.49(N/A,1)	49.46(N/A,1)
NASH	Reference Sequence(GG/CC)	0.24(0.19,4)	17.51(17.02,4)
	CYP2C19*17(GG/CT)	1.97(N/A,1)	23.45(N/A,1)

Data are presented as mean (SD, No.).

Table 4. Metabolic AUC ratio of Midazolam in CYP3A4 polymorphic subjects

	Genotype	Plasma AUC ratio (SD, No.)	Urine AUC ratio (SD, No.)
Control	Reference Sequence(AA)	3.84(1.15,14)	20.48(10.66,14)
	CYP3A4*1b(AG)	3.36(0.71,2)	10.24(N/A,1)
Steatosis	Reference Sequence(AA)	3.56(0.48,4)	16.97(11.35,4)
	CYP3A4*1b(AG)	2.46(0.41,3)	24.90(5.54,3)
NASH	Reference Sequence(AA)	2.45(0.89,4)	20.61(8.85,4)
	CYP3A4*1b(AG)	1.63(N/A,1)	15.22(N/A,1)

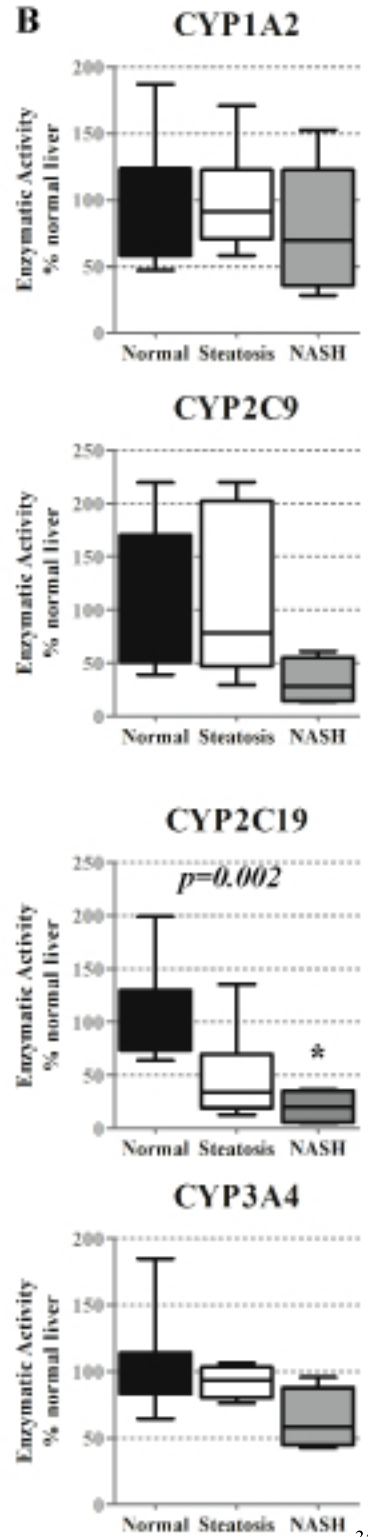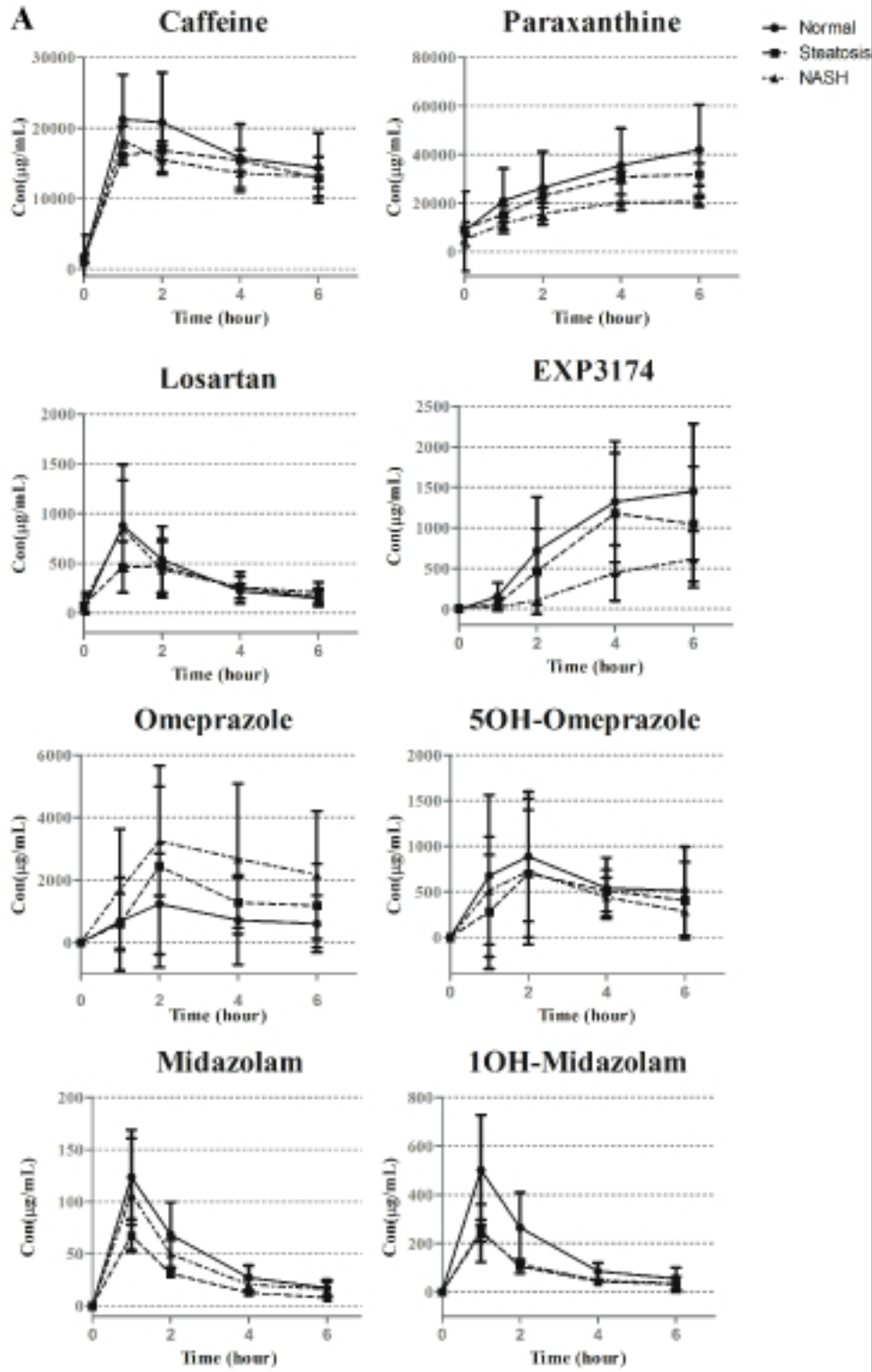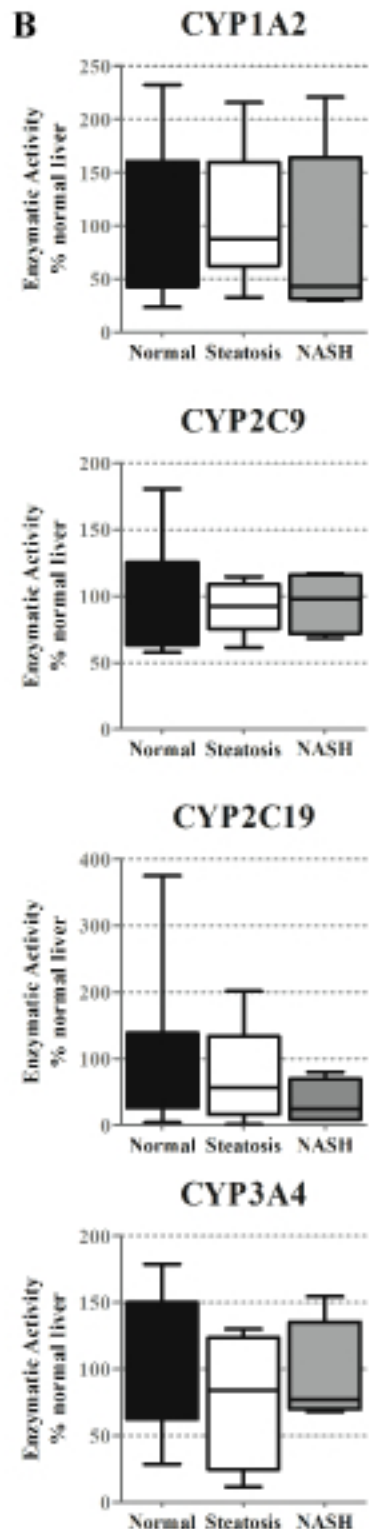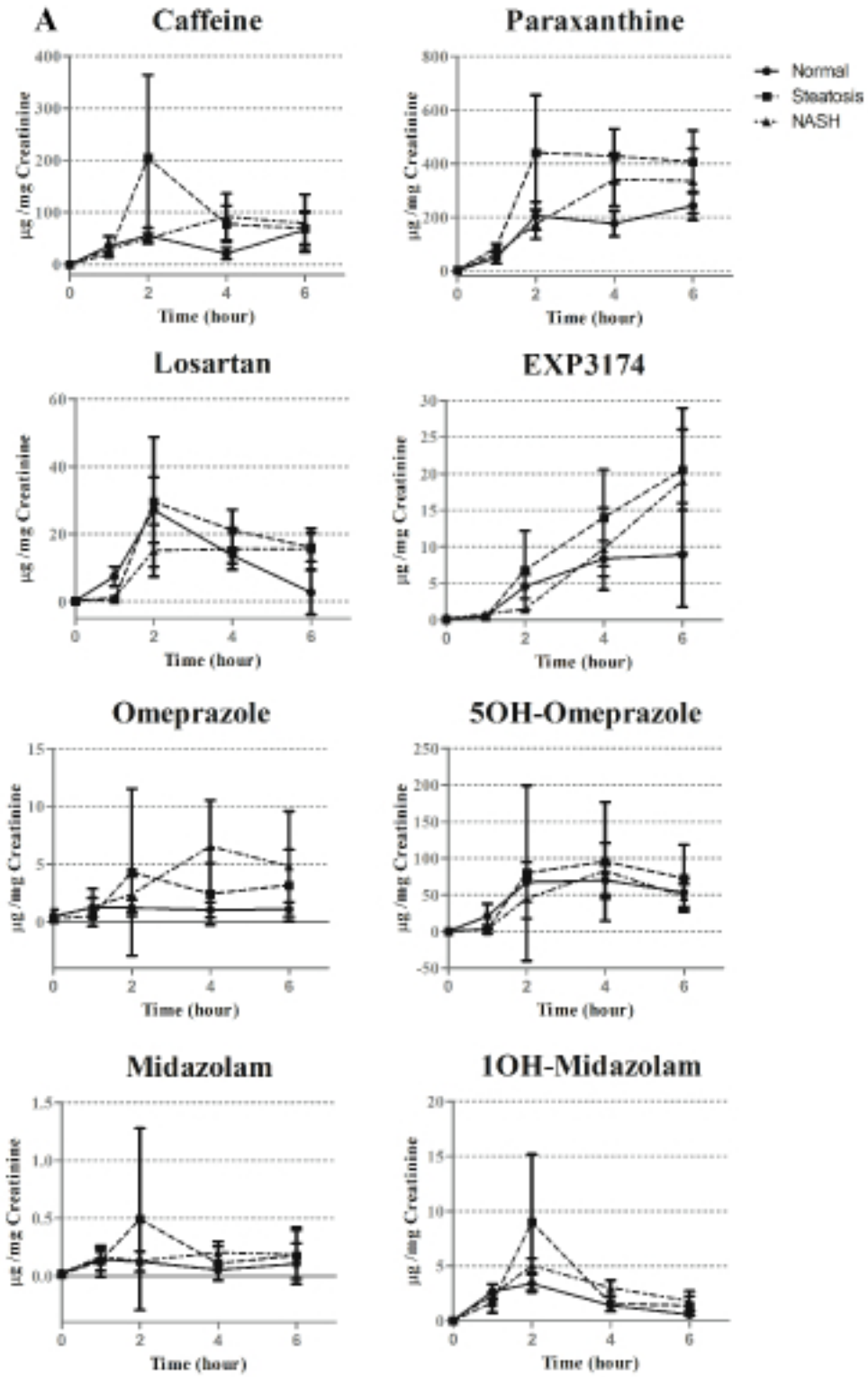Data are presented as mean (SD, No.).

Figure 1

A

Caffeine

Paraxanthine

Losartan

EXP3174

Omeprazole

5OH-Omeprazole

Midazolam

1OH-Midazolam

— Normal
—■— Steatosis
—▲— NASH

B

CYP1A2

CYP2C9

CYP2C19

p=0.002

CYP3A4

35

Figure 2

Panel A: Caffeine, Paraxanthine, Losartan, EXP3174, Omeprazole, 5OH-Omeprazole, Midazolam, 1OH-Midazolam — urinary concentration (µg /mg Creatinine) vs Time (hour) for Normal, Steatosis, and NASH groups.

Panel B: CYP1A2, CYP2C9, CYP2C19, CYP3A4 — Enzymatic Activity (% normal liver) for Normal, Steatosis, and NASH.

Figure 3.