Altered Regulation of Hepatic Efflux Transporters Disrupts Acetaminophen Disposition in Pediatric Nonalcoholic Steatohepatitis


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Abbreviations: APAP, N-acetyl-para-aminophenol; APAP-gluc, N-acetyl-para-aminophenol glucuronide; APAP-sulf, N-acetyl-para-aminophenol; MRP2, multidrug resistance-associated protein 2; MRP3, multidrug resistance-associated protein 3; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis.
Abstract

Non-alcoholic fatty liver disease (NAFLD) is the most common chronic liver disease and represents a spectrum of liver pathologies that include simple hepatic steatosis and the more advanced, nonalcoholic steatohepatitis (NASH). The current study was conducted to determine whether pediatric NASH also results in altered disposition of acetaminophen (APAP) and its two primary metabolites, APAP-sulfate and APAP-glucuronide. Pediatric patients with hepatic steatosis (N= 9) or NASH (N=3), and healthy patients (N=12), were recruited in a small pilot study design. All patients received a single 1000 mg dose of APAP. Blood and urine samples were collected at 1, 2, and 4h post dose and APAP and APAP metabolites were determined by HPLC. Moreover, human liver tissues from patients diagnosed with various stages of NAFLD were acquired from the Liver Tissue Cell Distribution System (LTCDS) to investigate the regulation of the membrane transporters, MRP2 and MRP3. Patients with the more severe disease (NASH) had increased serum and urinary levels of APAP-glucuronide along with decreased serum levels of APAP-sulfate. Moreover, an induction of hepatic MRP3 and altered canalicular localization of the biliary efflux transporter, MRP2, describes the likely mechanism for the observed increase in plasma retention of APAP-glucuronide, whereas altered regulation of sulfur activation genes may explain decreased sulfonation activity in NASH. APAP-glucuronide and APAP-sulfate disposition is altered in NASH and is likely due to hepatic membrane transporter dysregulation as well as altered intracellular sulfur activation.
Nonalcoholic fatty liver disease (NAFLD) is the most prevalent chronic liver disease in the US and many other industrialized nations (Lomonaco et al., 2013; Wieckowska and Feldstein, 2008). Although initially described almost 30 years ago, the true scope of the disease has only recently been understood. NAFLD encompasses a number of progressive disease stages, linked by the presence of hepatocellular lipid accumulation. The full spectrum of the disorder ranges from simple steatosis to nonalcoholic steatohepatitis (NASH), which may further progress to end stage liver diseases such as cirrhosis, and hepatocellular carcinoma (Lomonaco et al., 2013; Starley et al., 2010).

Given the disease’s close association with metabolic morbidities such as obesity and insulin resistance (IR), NAFLD is often regarded as the hepatic manifestation of the metabolic syndrome. The number of Americans with these three conditions (NAFLD, IR, obesity) has increased dramatically in the last few decades, and is still on the rise. In 2008, the prevalence of obese adults [body mass index (BMI) > 30 kg/m²] in the US was approximately 32% and is projected to reach more than 50% by 2030 (Flegal et al., 2010; Wang et al., 2008). Similarly, the prevalence of diabetes in the U.S. is estimated to increase from 14% in 2007 up to 33% by 2050 and therefore concomitantly increasing the incidence of metabolic comorbidities such as NAFLD (Boyle et al., 2010).

While NAFLD cases are largely benign and present as simple steatosis, the progression to NASH significantly increases the risk for hepatic morbidity and mortality. NASH is the most common cause of cryptogenic cirrhosis, and was reported to be the most likely etiologic risk factor in developing hepatocellular carcinoma (Preiss and Sattar, 2008; Rahimi and Landaverde, 2013). The prevalence of NAFLD is estimated to be 6-30% worldwide, whereas NASH is
reported to affect as high as 12.2% of the general population (Lomonaco et al., 2013; Rahimi and Landaverde, 2013). Moreover, once thought of as primarily an adult disease, NAFLD is now known to afflict children as well.

It is now recognized that NAFLD is an important pediatric liver disorder (Lavine et al., 2010; Patton et al., 2006). Like adults, as the prevalence rates for obesity rise among pediatric patients, so does the prevalence of NAFLD. It is estimated that 17% of children in Western society are overweight and among them, up to 80% may also have NAFLD (Giorgio et al., 2013). In pediatric patients, the prevalence of NAFLD is estimated to be 9.6%, and the rate is higher among adolescents (17.3%) than infants (0.7%) (Bozic et al., 2013; Patton et al., 2006; Schwimmer et al., 2006).

It has been well documented that NAFLD and especially NASH alter the expression of proteins involved in drug metabolism and disposition, particularly membrane transporters and biotransformation enzymes (Fisher et al., 2009b; Gomez-Lechon et al., 2009; Hardwick et al., 2011; Lake et al., 2011). We have previously reported that experimental NASH in a rat model alters drug transporter expression, resulting in a significant shift in the disposition of the acetaminophen (APAP) metabolite acetaminophen-glucuronide (APAP-gluc), from bile to blood and urine (Lickteig et al., 2007a). Moreover, a recent study has reported altered APAP-gluc disposition in children; however, a mechanism for these observations was left to speculation (Barshop et al., 2011).

The purpose of the current study was to determine the effects of pediatric NASH on the disposition of APAP and its primary metabolites, APAP-sulfate (APAP-sulf) and APAP-gluc. In addition, the regulation of two hepatic membrane transporters, MRP2 and MRP3, and intracellular sulfur activation were investigated in NASH as potential mechanisms for altered
APAP disposition. Using a small pilot study design, this investigation contributes to the current knowledge of NASH affecting drug disposition and offers an incentive for exploring these findings in a larger randomized-controlled setting.
Materials and Methods

**Materials** - Tris-HCL, EDTA, NaCl, glycerol, and nonidet P-40 were obtained from Sigma-Aldrich (St. Louis, MO).

**Clinical subjects** - Pediatric subjects (n=12) between the ages of 12 and 18 were recruited from a pool of NAFLD patients. All patients (non-NAFLD exempt) had undergone a prior liver biopsy as part of routine patient care. These biopsies were used to establish two distinct groups of patients (simple steatosis and NASH) based on the severity of three characteristics of NAFLD, including steatosis, fibrosis, and inflammation. In addition, pediatric subjects (n=12) between the ages of 12 and 18 were recruited from a panel of non-NAFLD patients. Many of these subjects were patients with constipation or abdominal pain without NAFLD. All patients and their legal guardians were approached during regular office visits, and informed consent received. To participate in the current study, each subject passed a screening evaluation based on medical history and physical examination. Subjects also had to meet basic inclusion/exclusion criteria highlighted below:

**Inclusion criteria:**

- Liver biopsy indicating either simple steatosis or NASH
- Age 12-18
- Informed consent and assent

**Exclusion criteria:**

- History of significant alcohol consumption (> 20 g/d)
- Clinical or histological evidence of cirrhosis
- Evidence of other chronic liver disease (ie Dubin-Johnson syndrome)
• Presence of the hepatitis B virus surface antigen or hepatitis C virus antibodies
• Use of drugs historically associated with NAFLD
• Use of anti-NAFLD drugs in the three months prior to enrollment in this study
• Pregnancy or breastfeeding
• History of renal dysfunction
• Other disease or conditions considered by the physician to be significant (e.g., cardiovascular disease)

Inclusion and exclusion criteria were applied to non-NAFLD participants as well, with the obvious exception of NAFLD diagnosis. Non-NAFLD patients were lean, and had no significant medical history. The study and all study procedures were approved by the University of Arizona Institutional Review Board (#00004218) prior to study commencement.

Visit procedures - Subjects arrived at the Clinical and Translational Science Center at the University of Arizona Medical Center on the morning of the study following an overnight fast. Subjects were advised to avoid any product containing APAP for at least three days prior to study participation. Approximately 7cc of blood were collected in 2 separate tubes, one of which was immediately sent for serum biochemistry analysis. The second blood sample and an initial urine sample were used as blank samples in the quantification of serum APAP levels by HPLC. Following these baseline collections of blood and urine, subjects were given one oral dose of 1000 mg APAP (McNeil Consumer Healthcare; Fort Washington, PA). Subjects were allowed access to food and water during the four hour study. Blood and urine were collected at 1, 2, and 4 hours by the attending nursing staff. Approximately 7 cc of blood were collected at each time
point into a serum-separator vacutainer tube (BD; Franklin Lakes, NJ). Samples were allowed to clot, and centrifuged to obtain the serum fraction. Each sample was subdivided into multiple aliquots to avoid excessive freeze/thaw cycles. The serum and urine samples for HPLC analysis were stored at -80° C for future analyses.

**Human Liver Samples** - Frozen and formalin-fixed, paraffin-embedded adult human liver tissue was obtained from the Liver Tissue Cell Distribution System (LTCDS) coordinated through the University of Minnesota, Virginia Commonwealth University and the University of Pittsburgh as described previously (Hardwick RN, 2011). Briefly, all samples were scored and categorized by a medical pathologist within the Liver Tissue Cell Distribution System according to a previously validated scoring rubric developed by Kleiner, et al. and pathology was then confirmed at the University of Arizona (Kleiner et al., 2005). Donor information, including the age and gender of the donors, has been published previously (Fisher et al., 2009a). The samples were diagnosed as either normal (n=20), steatotic (n=12), NASH with fatty liver (NASH fatty, n=11), and NASH without fatty liver (NASH not fatty/cirrhosis, n=11). For the purposes of this study, NASH fatty and NASH without fat were combined as one experimental group diagnosed as “NASH.” Those samples exhibiting >10% fatty infiltration of hepatocytes were staged as steatotic. Samples were diagnosed as NASH when >5% fatty infiltration of hepatocytes occurred with significant inflammation and fibrosis. These liver specimens were utilized for protein, mRNA, and immunohistochemical analyses described below.

**Tissue Preparations** - Whole cell lysate preparations of human liver tissue were prepared from tissue homogenized in NP-40 buffer (20 mM Tris HCl, 137 mM NaCl, 10% glycerol, 1% nonidet P-40, and 2 mM EDTA with 1 Protease Inhibitor Cocktail Tablet (Roche, Indianapolis, IN) per
25mL) at 4°C. Homogenized tissue was then agitated at 4°C for 2 hours, centrifuged at 10,000 x g for 30 minutes, and the supernatant transferred to a clean collection tube. Protein concentrations were determined using the Pierce BCA Protein Quantitation Assay (Thermo Scientific, Rockford, IL) per the manufacturer’s recommendations.

**MRP3 Immunoblot Analysis** - Whole cell lysate proteins (50 µg/well) were prepared in Laemmli Sample Buffer (Bio-Rad Laboratories, Hercules, CA) in non-reducing conditions (without β-mercaptoethanol or boiling) and separated by SDS-PAGE using 7.5% Tris-Glycine gels followed by transfer to polyvinylidene fluoride (PVDF) membranes. Membranes were blocked for one hour at room temperature using a 5% nonfat dry milk solution dissolved in phosphate buffered saline-tween (PBST). Following the membrane block, the membrane was incubated overnight in a primary antibody incubation (in 5% milk solution) using the following mouse monoclonal antibody raised against MRP3 protein: M3II-9, Abcam, Inc. (Cambridge, MA). An anti-mouse HRP-conjugated secondary (sc-2005, Santa Cruz Biotechnology, Santa Cruz, CA.) was used for detection and incubated for 1 h at room temperature. Quantification of relative protein expression was determined using image processing and analysis with Image J software (NIH, Bethesda, MD) and normalized to total pan-Cadherin (1:7000, Abcam, Inc., Cambridge, MA).

**MRP2 Liver Immunohistochemistry** - Immunohistochemical staining for all proteins was performed on formalin-fixed, paraffin-embedded (FFPE) human liver samples as described previously (Hardwick RN, 2011). Briefly, tissue sections were de-paraffinized in xylene and re-hydrated in ethanol, followed by antigen retrieval in citrate buffer (pH 6.0). Endogenous
peroxidase activity was blocked with 0.3% (v/v) H₂O₂ in methanol for 20 minutes. Immunohistochemical staining for MRP2 was performed with the MACH3 staining kit (Biocare Medical, Concord, CA) per the manufacturer’s protocol. Samples were incubated in an MRP2 primary antibody (M2III-5, Kamiya Biomedical Company, Seattle, WA) solution overnight at 4°C. All slides were counterstained with hematoxylin (Sigma Aldrich, St. Louis, MO) following color development with Betazoid DAB (Biocare Medical, Concord, CA). All slides were imaged with a Nikon Eclipse E4000 microscope and a Sony Exwave DXC-390 camera.

**APAP and APAP Metabolite Quantification** - Acetaminophen (APAP) and its primary metabolites (APAP-glucuronide and APAP-sulfate) were analyzed in serum and urine under high performance liquid chromatography conditions followed by UV detection as previously described (Lickteig et al., 2007a).

**Microarray Expression Analysis of Sulfur Activation Genes** - Individual Affymetrix GeneChip Human 1.0 ST Arrays (Affymetrix, La Jolla, CA) were generated from purified mRNA for each liver sample as previously described (Lake et al., 2011). The expression of 33,252 annotated and unannotated genes among three diagnosis groups (normal, steatosis, and NASH) is available in the array data set which is accessible at the ArrayExpress public repository for microarray data (accession number E-MEXP-3291) (http://www.webcitation.org/5zyojNu7T).

**Statistical Analysis** - Statistical differences between patient groups at each time point were determined using a one-way analysis of variance followed by a Bonferroni post-hoc test. Immunoblot data were analyzed by a non-parametric trend analysis and described as box and
whisker plots. The level of significance was set at $p \leq 0.05$ for all analyses using Stata9 statistical software (Stata, College Station, TX).
Results

Patient Demographics and Serum Chemistry

Table 1 displays study participant demographics and the results of serum biochemistry tests taken at the time of the study. Male patients were more predominant than females, particularly within the simple steatosis and NASH patient groups. Median ages were also similar across groups. Analysis of hepatic function was measured, including total serum protein, serum albumin, conjugated (direct) and total bilirubin, alkaline phosphatase (ALP), aspartate transaminase (AST), alanine transaminase (ALT). No statistical difference was observed in all analytes measured except AST and ALT, which were significantly elevated in patients with NASH (Table 1). Moreover, the majority of NAFLD patients recruited on study (steatosis and NASH) were male and of a Hispanic racial background indicative of the local population (data not shown).

Serum and Urine APAP and APAP Metabolites in Pediatric NAFLD Patients

To determine the effects of NAFLD on APAP disposition, all study participants were given a single oral dose of APAP (1000 mg) followed by measurement of parent and APAP-gluc and APAP-sulf in serum and urine at 1, 2, and 4 hours post-dose. The results of the analysis are described in Figure 1. No difference was observed in APAP serum or urine levels between the three groups at any time point. The levels of APAP-sulf in serum tended to decrease at all time points measured in patients with NASH, but these data did not reach statistical significance (Figure 1A). In contrast, serum APAP-gluc levels in children with NASH were significantly increased above normal patients at one hour following administration, and remained elevated compared to patients in both the normal and steatosis groups over all time points measured; however, plasma AUC values are not significantly different across all three groups (Figure 1A).
The increase in serum APAP-gluc in NASH subjects was accompanied by a significant increase in urin... of patients with simple steatosis (Figure 1B). No change in APAP-sulf concentration was detected in the urine across all subjects.

Hepatic MRP3 and MRP2 Regulation in Human NASH Patients

To investigate a potential molecular mechanism responsible for the observed effects of NASH on APAP-gluc disposition, the regulation of hepatic membrane transporters, MRP3 and MRP2, was determined via Western blot analysis and immunohistochemistry, respectively. The expression of the basolateral efflux transporter, MRP3, is significantly elevated in the livers of patients with NASH (Figure 2). In contrast, the apical efflux transporter responsible for biliary efflux of APAP-gluc, MRP2, appears to be improperly localized away from the canalicular membrane of hepatocytes in NASH patients (Figure 3). This is evidenced by the lack of sharp, localized staining on the apical membrane (Figure 3, circled), which is in contrast to what is observed in healthy and steatosis subjects.

Sulfur Activation and Utilization Gene Expression.

To further determine the potential for altered SULT activity in vivo we utilized results from a previously validated and published microarray experiment in these same human samples to determine the expression of key players in the sulfur activation and utilization pathway (Lake et al., 2011). Results of the gene expression analysis are shown in Figure 4. Expression of the sulfur uptake transporter, SLC26A1, is significantly reduced in NASH liver compared to normal; however, expression of SLC26A2 was unchanged. In addition to extracellular uptake, sulfur may also be liberated from cysteine pools in the cell through the action of cysteine dioxygenase type 1
(CDO1) resulting in sulfite which is then converted to sulfate by sulfite oxidase (SUOX) (Feng et al., 2007; Markovich, 2001; Wilkinson and Waring, 2002). CDO1 and SUOX were both significantly down-regulated in NASH samples compared to normal suggesting that there is a decreased potential for sulfate activation from intracellular sources.
Discussion

The purpose of the current study was to investigate the effects of NASH on the disposition of APAP and its two primary metabolites, APAP-gluc and APAP-sulf, in a cohort of pediatric patients. Our results indicate that children with NASH tend to have increased retention of the metabolite, APAP-gluc, in systemic circulation along with increased excretion into the urine. Moreover, we identify the dysregulation of the hepatic membrane transporters, MRP2 and MRP3, as potential mechanism for these observations. However, a significant limitation of this study is the low number of NASH patients that were recruited on study, as well as the lack of ethnic, racial and sexual diversity within our patient pool, which was primarily male and of Hispanic background. Therefore, the validation and significance of these findings as a potential diagnostic to distinguish patients with NASH from those with milder disease will benefit from the inclusion of a larger randomized trial in the future. Moreover, the analyses of MRP2 and MRP3 expression were performed in liver samples obtained from primarily an adult population, whereas the age range of the clinical subjects tested is 12-18. Developmental patterns in transporter expression are known to exist; however, recent evidence demonstrates that, at least for MRP2 and MDR1, expression appears to stabilize prior to puberty (Mooij et al., 2014; Prasad et al., 2013), suggesting that the expression data presented in this manuscript may be an accurate representation of the expression profiles of the adolescents on study. Despite these limitations, the results gathered from this study warrant further consideration when administering pharmaceuticals to children with NAFLD as they may be at higher risk for developing adverse drug reactions as a result of aberrant drug disposition.

The metabolism and disposition of APAP has been heavily investigated and well characterized. Glucuronidation and sulfonation of APAP in the liver are the predominant
metabolic pathways, and account for 50-70% and 25-35% of acetaminophen, respectively (McGill and Jaeschke, 2013). Unlike APAP parent, APAP-gluc and APAP-sulf are considerably more polar and require the aid of membrane transporters for proper efflux and excretion from the body. In healthy livers, biliary excretion of the sulfate and glucuronide conjugates of APAP is predominantly mediated by MRP2, which is localized to the apical, or canalicular membrane of hepatocytes (Xiong et al., 2000). Sinusoidal excretion of the APAP-gluc metabolite from hepatocytes is predominantly mediated by MRP3 (Manautou et al., 2005) while MRP4, which is also expressed on the sinusoidal membrane, appears to mediate excretion of APAP-sulf metabolites (Zamek-Gliszczynski et al., 2006).

Despite the limited number of NASH patients, our findings are consistent with previous investigations. Specifically, we have previously reported the effects of experimental NASH on the disposition of APAP-gluc (Lickteig et al., 2007a). Similar to our observations in pediatric patients, NASH rodents demonstrated an increase in plasma and urine levels of APAP-gluc (Lickteig et al., 2007a). Moreover, these findings were coupled to decreased biliary excretion of APAP-gluc. This shift from bile to plasma disposition was presumably due to an induction of the hepatic sinusoidal membrane transporter, MRP3, which we also found to be induced in human NASH as described above. However, it is interesting to note that although biliary excretion of APAP-gluc was decreased, MRP2 protein was induced in experimental NASH (Lickteig et al., 2007a). This anomaly was later confirmed using a rat model of NASH where it was noted that Mrp2 localization was altered, which subsequently lead to altered ezetimibe disposition (Hardwick et al., 2012). These effects were suggested to be due to MRP2 being internalized from the canalicular membrane, which would consequently diminish MRP2-mediated excretion into the bile. Indeed, previous investigations have demonstrated Mrp2 is reversibly internalized into
intracellular compartments and mediated by the redox-sensitive balance of protein kinase C activation in experimental cholestasis (Mottino et al., 2002; Sekine et al., 2008; Sekine et al., 2011). Oxidative stress is a central feature of NASH pathogenesis and we have previously identified the activation of key redox-sensitive genes in both humans and rodents with NASH (Hardwick et al., 2010; Lickteig et al., 2007b). Together, our data confirm the original findings in the rodent model and suggest that MRP2 localization is altered in human NASH.

Barshop et al., previously reported that acetaminophen disposition is altered in pediatric patients afflicted with NAFLD. In this study, APAP-gluc was found to be slightly increased in both the plasma and urine of pediatric patients with NAFLD (Barshop et al., 2011). Our results are in agreement with these previous findings and demonstrate that the mechanistic features of transport function in pediatric NASH causes a functional disruption in APAP-gluc disposition. However, in contrast to our conclusions, the previous investigators allude to altered glucuronidation, presumably via disruption of enzymatic activity, as a mechanism for the observed increase in systemic APAP-gluc levels (Barshop et al., 2011). Although we did not investigate glucuronidation capacity in these patients, more recent findings have demonstrated that APAP glucuronidation is not altered in NASH subjects, suggesting that the observed effects of NASH on APAP-gluc disposition is less likely mediated by altered glucuronidation (Hardwick et al., 2013). In contrast, we argue that decreased MRP2 function (due to altered membrane localization) coupled to induction of MRP3 is the primary mechanism resulting in increased systemic exposure to APAP-gluc in these patients. However, glucuronidation capacity is known to be variable among children and therefore further characterization of APAP glucuronidation in children with NASH is needed for a more comprehensive conclusion.
In spite of the induction in MRP3 protein expression, we do not observe a concomitant increase in serum APAP-sulf levels following APAP administration in these NASH subjects. In contrast, we report a decreasing trend in serum APAP-sulf in NASH, which is consistent with previous findings in a rodent model dosed with APAP (Lickteig et al., 2007a). These results are also in parallel with previous data that demonstrate decreased pan-sulfotransferase activity in NASH despite the protein induction of several sulfotransferase isoforms in the disease (Hardwick et al., 2013). Moreover, decreased total sulfotransferase activity in humans with alcoholic and nonalcoholic liver disease was identified in an independent study (Yalcin et al., 2013). Together, these results are suggestive of disrupted cellular sulfur activation and utilization in NASH, which would ultimately limit the intracellular concentrations of the sulfonation cofactor, 3’-phosphoadenosine-5’-phosphosulfate (PAPS). Indeed, we demonstrate altered expression of genes critically involved in sulfur utilization in this study. Specifically, we identify decreased expression of the sulfur uptake transporter, SLC26A1, as well as deceased expression of CDO1 and SUOX, which are important in liberating intracellular sulfur pools from the amino acid cysteine (Feng et al., 2007; Wilkinson and Waring, 2002). Together, these results demonstrate decreased hepatic capacity to synthesize PAPS in NASH, which may partially explain the slight decrease in serum APAP-sulf observed.

It is worth noting that in addition to APAP-gluc being elevated when compared to normal, healthy clinical subjects, it is also elevated in NASH over patients with simple steatosis due to the mechanistic features of altered transporter function only present at the later stage of the disease. This highlights the possible utility of using serum APAP-gluc levels as a potential biomarker to be used as a non-invasive tool for distinguishing NASH from “not NASH”. Histological analysis of a liver biopsy still remains the gold standard in diagnosing NAFLD, as it
is able to assess steatosis, fibrosis, and inflammation, as well as changes in overall liver architecture (Wieckowska and Feldstein, 2008). However, this is an invasive procedure and impractical to use per standard of care. Moreover, current methods of distinguishing patients with steatosis from those with NASH lack the specificity and sensitivity to replace liver biopsy. While the majority of NAFLD diagnoses are currently made on the basis of elevated aminotransferase levels, normal serum aminotransferase tests can be seen in patients with both steatosis and NASH (Ipekci et al., 2003; Mofrad et al., 2003), which is consistent with our observations (Table 1). Furthermore, several investigators have reported that two-thirds of NASH patients may have normal aminotransferase levels at any given time (Delgado, 2008; Oh et al., 2008; Wieckowska and Feldstein, 2008), highlighting the need for a more effective, noninvasive means of diagnosing NASH. The clinical development of an APAP-gluc disposition test for NASH may help indicate at risk patients for diagnostic liver biopsies, or serve as a noninvasive means of tracking progression or treatment of the disease.

In conclusion, the results obtained from this preliminary pilot study demonstrate the potential for NAFLD to disrupt drug pharmacokinetics in children. Specifically, altered MRP2 localization and MRP3 induction appear to represent a primary mechanism for the increase in APAP-gluc in pediatric NAFLD patients. Although APAP-gluc is pharmacologically and/or toxicologically inactive, serious health risks may be imposed in the event of increased systemic exposure of a highly active metabolite in patients with NAFLD. Further understanding how NASH affects renal clearance mechanisms would also be highly beneficial in understanding how the disease affects all excretory routes and of course, utilizing a larger sample size in a randomized controlled setting is warranted for further verification of these findings and their clinical implications.
Authorship Contributions

Participated in research design: Canet, Merrell, Erickson, Cherrington.

Conducted experiments: Canet, Merrell, Hardwick, Bataille, Campion, Ferreira

Contributed new reagents: Manautou, A-Kader, Erickson, Xanthakos

Performed data analysis: Canet, Merrell and Cherrington.

Wrote or contributed to the writing of the manuscript: Canet, Merrell and Cherrington
References


Footnotes

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Mark J. Canet and Matthew D. Merrell both contributed equally to this work.

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Figure Legends

Figure 1: Plasma and Urine APAP, APAP-gluc, and APAP-sulf in Pediatric NAFLD. Plasma (1A) and urine (1B) concentrations of APAP, APAP-gluc, and APAP-sulf following a single oral administration of 1000 APAP. Samples were collected at 1, 2, and 4h post-dosing and measured by HPLC-UV. Area under the plasma vs. time curve (AUC) for APAP, APAP-gluc, and APAP-sulf were calculated for all three diagnosis stages and are provided as an insert to panel A. The p-values provided for the AUC data represent the calculated ANOVA p-value across all three diagnosis stages. * p < 0.05 healthy patients compared to NASH patients; † p < 0.05 patients with simple steatosis compared to NASH.

Figure 2: Hepatic MRP3 Protein Induction in NASH. MRP3 protein was measured via immunoblot analysis from human liver samples obtained from human patients with ages ranging from 16-70 that were diagnosed as being normal, steatotic (NAFLD), or having NASH as described in the methods section. * p < 0.05 healthy patients compared to NASH patients; † p < 0.05 patients with simple steatosis compared to NASH.

Figure 3: Hepatic MRP2 Localization in Patients with NASH. Immunohistochemistry was used to detect and visualize MRP2 protein localization within normal, steatosis, and NASH liver. The red circles indicate regions of perturbed localization of MRP2 on the canalicular membrane. Negative controls (performed without primary antibody) are included to demonstrate positive,
membrane staining. All images were taken at 100x magnification and are representative images of multiple IHC sample analyses.

Figure 4: Normalized Gene Expression of Sulfur Activation and Utilization Pathways. Hepatic gene expression of enzymes and transporters involved in the activation and utilization of sulfur are shown in human liver samples diagnosed as normal, steatotic, and NASH. Gene expression data was mined from a previously validated and published microarray experiment performed in a subset of aforementioned human liver samples [16]. Data was normalized to the median of the normal diagnostic category and presented as normalized gene expression. * p < 0.05 healthy patients compared to NASH patients.
Table 1: Study Participant Demographics and Blood Chemistry

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*p <0.05 from NASH compared to healthy and simple steatosis.

nm: not measured
Figure 1

**A**

APAP

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<th>Diagnosis</th>
<th>AUC (nmol/h·mL¹)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>141.1 ± 11.5</td>
<td></td>
</tr>
<tr>
<td>Steatosis</td>
<td>134.2 ± 19.2</td>
<td>0.69</td>
</tr>
<tr>
<td>NASH</td>
<td>115.0 ± 17.6</td>
<td></td>
</tr>
</tbody>
</table>

![APAP - Normal, Steatosis, NASH](chart_AAPAP)

Time (h)

**B**

APAP

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>AUC (nmol/h·mL¹)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>143.0 ± 17.0</td>
<td></td>
</tr>
<tr>
<td>Steatosis</td>
<td>117.19 ± 15.7</td>
<td>0.09</td>
</tr>
<tr>
<td>NASH</td>
<td>207.7 ± 33.0</td>
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</table>

![APAP - Normal, Steatosis, NASH](chart_BAPAP)

Time (h)

**APAP-Gluc**

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>AUC (nmol/h·mL¹)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>46.4 ± 5.0</td>
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</tr>
<tr>
<td>Steatosis</td>
<td>42.1 ± 10.3</td>
<td>0.32</td>
</tr>
<tr>
<td>NASH</td>
<td>23.0 ± 9.1</td>
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</tbody>
</table>

![APAP-Gluc - Normal, Steatosis, NASH](chart_AAPAPGluc)

Time (h)

**APAP-Sulf**

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>AUC (nmol/h·mL¹)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>149.6 ± 11.5</td>
<td></td>
</tr>
<tr>
<td>Steatosis</td>
<td>139.7 ± 19.2</td>
<td>0.69</td>
</tr>
<tr>
<td>NASH</td>
<td>121.0 ± 17.6</td>
<td></td>
</tr>
</tbody>
</table>

![APAP-Sulf - Normal, Steatosis, NASH](chart_AAPAPSulf)

Time (h)
Figure 2

MRP3 Protein

![Box plot showing relative protein expression for Normal, Steatosis, and NASH groups.](image)

![Western blot images for MRP3 and Pan-cadherin across Normal, Steatosis, and NASH groups.](image)
Figure 3

Normal  Steatosis  NASH

Negative Control (Normal)  Negative Control (NASH)
Figure 4

Box plots showing normalized gene expression levels for SLC26A1/SAT-1, SLC26A2/DTDST, CDO1, and SUOX in normal, steatosis, and NASH conditions.