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# Altered UDP-Glucuronosyltransferase (UGT) and Sulfotransferase (SULT) Expression and Function during Progressive Stages of Human Nonalcoholic Fatty Liver Disease

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**Running Title:** UGT and SULT enzyme expression in human NAFLD

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**Abbreviations:** UGT, UDP-glucuronosyltransferase; SULT, sulfotransferase; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; APAP, acetaminophen; PAPS, 3'-phosphoadenosine-5'-phosphosulfate; APS, 3'-adenosine-phosphosulfate; PAPSS1, PAPS synthase 1; PAPSS2, PAPS synthase 2

**Abstract**

The UDP-glucuronosyltransferases (UGTs) and sulfotransferases (SULTs) represent major Phase II drug-metabolizing enzymes that are also responsible for maintaining cellular homeostasis by metabolism of several endogenous molecules. Perturbations in the expression or function of these enzymes can lead to metabolic disorders and improper management of xenobiotics and endobiotics. Nonalcoholic fatty liver disease (NAFLD) represents a spectrum of liver damage ranging from steatosis to nonalcoholic steatohepatitis (NASH) and cirrhosis. Because the liver plays a central role in the metabolism of xenobiotics the purpose of the current study was to determine the effect of human NAFLD progression on the expression and function of UGTs and SULTs in normal, steatosis, NASH (fatty) and NASH (not fatty/cirrhosis) samples. We identified upregulation of UGT1A9, 2B10, and 3A1 and SULT1C4 mRNA in both stages of NASH, whereas UGT2A3, 2B15, and 2B28 and SULT1A1, 2B1, and 4A1 and PAPSS1 were increased in NASH (not fatty/cirrhosis) only. UGT1A9, 1A6 and SULT1A1, 2A1 protein levels were decreased in NASH; however SULT1C4 was increased. Measurement of the glucuronidation and sulfonation of acetaminophen revealed no alterations in glucuronidation; however, SULT activity was increased in steatosis compared to normal samples, but then decreased in NASH compared to steatosis. In conclusion, the expression of specific UGT and SULT isoforms appears to be differentially regulated, whereas sulfonation of APAP is disrupted during progression of NAFLD.

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## Introduction

The liver is regarded as the primary organ of drug metabolism and utilizes two categories of enzymes to metabolize xenobiotics in an effort to facilitate their removal from the body. These consist of Phase I enzymes which perform oxidative, reductive, or hydrolytic reactions that expose or introduce a functional group on a xenobiotic, and Phase II conjugative enzymes which lead to the addition of bulky, generally more water-soluble entities directly onto a xenobiotic or to a product of Phase I metabolism. The importance of the liver at the center of drug metabolism is exemplified by the multiplicity and redundancy of the enzymes that it expresses. Normally these enzymes work to aid in the removal of xenobiotics from the body thus reducing the potential for toxicity, but can sometimes lead to formation of a toxic metabolite (Zamek-Gliszczynski et al., 2006;King et al., 2000;Gamage et al., 2006). The occurrence of liver disease in an individual can alter the expression and function of these enzymes and complicate the drug metabolism process leading to either inadequate processing of xenobiotics thereby potentiating their effects in the body, enhanced bioactivation to toxic metabolites, or accelerated metabolism with the potential to reduce therapeutic efficacy.

Nonalcoholic fatty liver disease (NAFLD) is a chronic, progressive liver disease that begins as steatosis and can progress to nonalcoholic steatohepatitis (NASH) and even cirrhosis (Sanyal, 2011;Marra et al., 2008). NAFLD originates as simple steatosis which is characterized by accumulation of lipid droplets in >5% of hepatocytes and is largely considered benign but not quiescent (Marra et al., 2008). Steatosis may remain benign for several years; however, once progression to NASH occurs, further progression to cirrhosis is believed to be accelerated (Rubinstein et al., 2008). Progression to the more severe state of NASH is proposed to occur by several different mechanisms that ultimately result in significant liver damage in the form of greater lipid accumulation, inflammation, oxidative stress, hepatocellular damage, and varying

degrees of fibrosis (Marra et al., 2008;Brunt and Tiniakos, 2010). NAFLD has been estimated to affect 17-40% of the adult population, whereas the prevalence of NASH is estimated to range anywhere from 5.7-17% (Ali and Cusi, 2009;McCullough, 2011). Alarmingly, it is believed that 15-25% of patients with NAFLD will develop cirrhosis, of which 30% will die within ten years following diagnosis (McCullough, 2011;Rubinstein et al., 2008). Due to its progressive nature and its appreciable effects on liver histopathology, NAFLD is poised to have a significant impact on xenobiotic metabolism. Our laboratory has endeavored to understand the effect of NAFLD on drug metabolism in an effort to predict the potential for toxicity or altered therapeutic effect in patients. Previous investigations have identified alterations in the expression and function of cytochrome P450 enzymes as well as diminished glutathione transferase function in a bank of human tissues representing progressive stages of NAFLD (Fisher et al., 2009;Hardwick et al., 2010). However, little is known regarding the effect of human NAFLD on the Phase II enzymes UDP-glucuronosyltransferases (UGTs) or sulfotransferases (SULTs) (Merrell and Cherrington, 2011).

The UGTs are perhaps the most important family of Phase II enzymes responsible for metabolizing approximately 40-70% of drugs (Jancova et al., 2010). They are divided into four families: UGT1, 2, 3 and 8 with 1 and 2 being the most prominent in drug metabolism; however, not all isoforms are expressed in the liver (Bock, 2010;King et al., 2000;Jancova et al., 2010). UGTs reside in the endoplasmic reticulum and catalyze the conjugation of primarily glucuronic acid (with the exception of the UGT3 and 8 families) to xenobiotics, bile acids, steroid hormones, bilirubin, and eicosanoids using the cofactor uridine 5'-diphosphoglucuronic acid (UDP-GlcUA) (King et al., 2000;Meech and Mackenzie, 2010;Bock, 2010). The SULT enzymes are another important group of Phase II enzymes that consists of 4 families in humans: SULT1, 2, 4, and 6 (Jancova et al., 2010). Drug metabolizing SULTs are located in the cytosol and catalyze the transfer of sulfonate to xenobiotics, bile acids, steroid hormones, and

neurotransmitters via the cofactor 3'-phosphoadenosine-5'-phosphosulfate (PAPS) (Gamage et al., 2006;Zamek-Gliszczynski et al., 2006). The SULTs tend to dominate at low concentrations due to higher affinity, and the UGTs prevail at high substrate concentrations which are saturating for SULTs, or when the availability of the SULT cofactor, PAPS, is exhausted (Zamek-Gliszczynski et al., 2006). In the current study, we have determined the effect of human NAFLD progression on the expression and function of several UGT and SULT isoforms.



## Materials and Methods

*Materials.* Tris, ethylenediaminetetraacetic acid (EDTA), potassium chloride (KCl), sodium pyrophosphate, potassium phosphate, Brij 58 (polyethylene glycol hexadecyl ether polyoxyethylene (20) cetyl ether), uridine 5'-diphosphoglucuronic acid (UDP-GlcUA), $MgCl_2$, acetaminophen (APAP), perchloric acid, 3'-phosphoadenosine 5'-phosphosulfate (PAPS), dithiothreitol (DTT), bovine serum albumin (BSA), HPLC-grade methanol, HPLC-grade water, and acetic acid were obtained from Sigma Aldrich (St. Louis, MO).

*Human Liver Samples.* Frozen adult human liver tissue was obtained from the NIH-funded Liver Tissue Cell Distribution System (LTCDS) coordinated through the University of Minnesota, Virginia Commonwealth University and the University of Pittsburgh. All samples were scored and categorized by a medical pathologist within the Liver Tissue Cell Distribution System according to the NAFLD Activity Score system developed by Kleiner, et al., followed by confirmation via histological examination at the University of Arizona (Kleiner et al., 2005). Donor information, including age and gender has been previously published for most samples (Fisher et al., 2009). Information for additional samples that have recently been added to the tissue bank can be found in Supplemental Data Table 1. The samples were diagnosed as either

normal (n=20), steatotic (n=12), NASH with fatty liver (NASH fatty, n=16), and NASH without fatty liver (NASH not fatty/cirrhosis, n=20). Samples exhibiting >10% fatty infiltration of hepatocytes were considered steatotic, whereas those with >5% fatty infiltration of hepatocytes in addition to significant inflammation and fibrosis were categorized as NASH (fatty). Samples were categorized as NASH (not fatty) when fatty deposits within hepatocytes were reduced <5% and accompanied by more marked inflammation and fibrotic branching. Representative histology images have been published previously (Hardwick et al., 2010;Hardwick et al., 2011;Fisher et al., 2009).

*Total RNA Isolation.* Total RNA was isolated from human liver tissue using RNAzol B reagent (Tel-Test Inc., Friendswood, TX) per the manufacturer's protocol. RNA integrity was confirmed by ethidium bromide staining following agarose gel electrophoresis. RNA concentrations were determined by UV spectrophotometry.

*Quantigene Plex 2.0 assay for mRNA quantification.* All reagents for analysis including lysis buffer, amplifier/label probe diluent and substrate solution were supplied in the QuantiGene Plex 2.0 assay kit (Affymetrix, Santa Clara, CA). Total RNA was mixed with master mix containing lysis mixture, blocking reagent, capture beads and 2.0 specific probeset, in a hybridization plate. After about 18 hours of hybridization at 54°C in Vortemp 56 (Labnet International, Woodbridge, NJ), the contents were transferred to a flat bottom magnetic plate. The washing at this step, and all subsequent washings were performed on a Bioplex Pro II wash station (Biorad, Hercules, CA). The hybridization complex beads were then incubated with preamplifier, amplifier and label probe, for one hour each, followed by incubation with streptavidin phycoerythrin (SAPE) for 30 min. After incubation with SAPE, the plate was washed with SAPE wash buffer followed by resuspension of the beads. The resulting fluorescence was read on a

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Bioplex multiple array reader system (Biorad, Hercules, CA). The raw data was obtained from Bioplex Manager 5.0 software.

*Cellular Fractionation for Protein Analysis.* Cytosolic and microsome fractions of human liver tissue were prepared by homogenization of ~300 mg of tissue in 3 mL of buffer containing 50 mM Tris-HCl (pH 7.4), 1 mM EDTA and 154 mM KCl with 1 Complete Protease Inhibitor Cocktail tablet (Roche, Indianapolis, IN) per 25mL at 4°C. Homogenates were centrifuged at 10,000 x g for 30 minutes. The supernatant was transferred to clean centrifuge tubes and the centrifugation step repeated. The resulting supernatant was then transferred to clean ultracentrifuge tubes and the samples were centrifuged at 100,000 x g for 70 minutes. The supernatant was retained as the cytosolic fraction. The remaining pellet was re-suspended in a buffer containing 100 mM sodium pyrophosphate (pH 7.4) and 1 mM EDTA. The samples were then centrifuged at 100,000 x g for 70 minutes and the supernatant was discarded. The remaining pellet was re-suspended with minimal sonication on ice in a buffer containing 10 mM potassium phosphate (pH 7.4), 1 mM EDTA, and 20% glycerol, this re-suspension was retained as the microsome fraction. Crude membrane fractions of human liver tissue were prepared by homogenization of ~250 mg of tissue in 2.5 mL buffer containing 0.1 M Tris-HCl (pH 7.4) and 1 Complete Protease Inhibitor Cocktail tablet (Roche, Indianapolis, IN) per 25 mL. Samples were transferred to clean ultracentrifuge tubes and centrifuged at 100,000 x g for 60 minutes. The supernatant was discarded and the remaining pellet re-suspended in Tris buffer. The re-suspension was retained as the crude membrane fraction. Protein concentrations for all cellular fractions were determined using the Pierce BCA protein quantitation assay (Thermo Fisher Scientific, Waltham, MA) per the manufacturer's instructions.

*Immunoblot Analysis.* Microsomal (UGTs-20 µg/well) or cytosolic (SULTs-20 µg/well) proteins from all samples were separated by SDS-polyacrylamide electrophoresis on 7.5% Tris-HCl gels

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and transferred to polyvinylidene difluoride membranes overnight. Rabbit polyclonal antibodies against UGT1A1 and 1A6 were obtained from Abcam, Inc. (Cambridge, MA) as well as mouse monoclonal antibodies against UGT2B10 and SULT1A1. A mouse polyclonal antibody was obtained from Abnova (Taipei, Taiwan) to detect UGT1A9. SULT1C4 and 2A1 were detected using rabbit polyclonal antibodies obtained from Abnova (Taipei, Taiwan) and Santa Cruz Biotechnology (Santa Cruz, CA), respectively. Pan-Cadherin was detected using a rabbit polyclonal antibody acquired from Abcam, Inc. (Cambridge, MA) and used as a control protein for microsomal proteins (UGTs). Goat polyclonal antibodies against ERK 1 (sc-93) and ERK 2 (sc-154) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA), and used to detect Total ERK which served as a control protein for cytosolic proteins (SULTs). Primary antibody incubations were performed overnight at 4°C followed by secondary detection the next day. Quantification of relative protein expression was determined using image processing and analysis with Image-J software (National Institutes of Health, Bethesda, MD) and normalized to pan-Cadherin.

*Analysis of UGT and SULT Activity Using Acetaminophen as a Probe Substrate.* Hepatic glucuronidation and sulfation of acetaminophen (APAP) was performed as previously described (Manautou et al., 1996;Reisman et al., 2009) with slight modifications. Glucuronidation was assessed by adding 50 µl of 0.05% Brij58 (polyethylene glycol hexadecyl ether polyoxyethylene (20) cetyl ether) to 250 µg of hepatic crude membrane protein in glass test tubes. Solutions of 25 mM uridine 5'-diphosphoglucuronic acid (UDP-GlcUA) and 50 mM $MgCl_2$ in 0.1 M sodium phosphate buffer (pH 7.8) were added to the samples and then pre-incubated at room temperature for 10 minutes. Upon addition of APAP, tubes were incubated in a 37°C water bath for 60 minutes. The final 500 µl reaction volume contained 0.5 mg/ml crude membrane protein, 10 mM $MgCl_2$, 5 mM APAP and 4 mM UDP-GlcUA in 0.1 M sodium phosphate buffer. Reactions were stopped by addition of 50 µl of 6% perchloric acid. Samples were chilled on ice and

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centrifuged at 3000 x g for 10 minutes at 4°C. 50 µl of collected supernatants were injected onto the HPLC for analysis. APAP-glucuronide (APAP-GLUC) formation was confirmed by conducting control incubations lacking APAP, crude membrane proteins, or UDP-GlcUA in which no APAP-GLUC metabolite was formed. Quantification of the APAP-GLUC peak was achieved by comparing peak areas of APAP-GLUC metabolite with an authentic APAP-GLUC standard (McNeil-PPC, Inc, Fort Washington, PA) and expressed as nmol APAP-GLUC/min/mg protein.

Sulfonation of APAP was performed by adding 125 µl 3'-phosphoadenosine 5'-phosphosulfate (PAPS) and 50 µl of dithiothreitol (DTT) containing 0.5% bovine serum albumin (BSA) in 0.1 M sodium phosphate buffer (w/v, pH 7.8) to empty borosilicate glass test tubes. 250 µg of cytosolic protein was added to each tube and diluted with 0.1 M sodium phosphate buffer to a total volume of 350 µl. Samples were placed in a 37°C water bath and 50 µl of APAP solution was added and mixed gently. Total reaction volume (400 µl final volume) contained 0.625 mg/ml cytosolic protein, 1 mM APAP, 8 mM DTT, 0.0625% BSA, and 0.1 mM PAPS. Reactions were stopped after 120 minutes by adding 400 µl of ice cold methanol. Samples were then centrifuged at 3000 x g for 10 minutes at 4°C and 50 µl of collected supernatant was analyzed by HPLC. APAP-sulfate (APAP-SULF) formation was confirmed by conducting control incubations lacking either APAP, cytosolic proteins, or PAPS in which no APAP-SULF metabolite was formed. Quantification of the APAP-SULF peak was achieved by comparing peak areas of APAP-SULF metabolite with an authentic APAP-SULF standard (McNeil-PPC, Inc, Fort Washington, PA) and expressed as pmol APAP-SULF/min/mg protein.

All HPLC analyses were performed using a Shimadzu LC-6AD pump with a SPD-20A UV-Vis detector (Shimadzu Scientific Instruments, Inc, Columbia, MD) at 254nm. A 250 x 4.6mm Ultrasphere C18 column with 5 µm particle size (Beckman, Brea, CA) was used. The flow of

mobile phase (12.5% methanol: 1% acetic acid in water) was maintained at a rate of 1.2 mL/min.

*Statistical Analysis.* Gene expression data was normalized to the housekeeping gene, GAPDH. To achieve normality of the data following GAPDH normalization, all data were log transformed. To determine significant changes in individual gene expression, the log transformed mRNA data were analyzed by ANOVA followed by the Tukey Honest Significant Difference (HSD) test. A Relative protein levels and enzyme activity data were not transformed; however all data were analyzed by an ANOVA followed by the Tukey HSD. A significance level of $p \leq 0.05$ was used for all statistical analyses.

**Results**

*Gene Expression of UGT and SULT Isoforms during Progression of Human NAFLD.* The mRNA expression of several UGT and SULT isoforms was determined by an Affymetrix Plex 2.0 Assay in normal, steatosis, NASH (fatty) and NASH (not fatty) human samples and expressed in Figures 1 and 2, respectively. UGT isoforms investigated included 1A1, 1A3, 1A4, 1A6, 1A9, 2B4, 2B7, 2B10, 2B15, 2B17, 2B28, 3A1, and 3A2. Overall, expression of many UGT genes tended to decrease in steatosis though these changes were not significant. UGT1A1 and 2B28 were significantly up-regulated in NASH (not fatty) samples compared to steatosis, whereas UGT1A9 and 2B10 were elevated in both stages of NASH compared to normal and steatosis. UGT3A1 gene expression was increased in both stages of NASH compared to steatosis. UGT2A3 expression was increased in NASH (not fatty) samples compared to normal; however, 2B15 was elevated in NASH (not fatty) compared to both normal and steatosis.

SULT and related genes investigated included 1A1, 1A2, 1B1, 1C2, 1C4, 1E1, 2A1, 2B1, 4A1, the pseudogene 1D1P, PAPSS1, and PAPSS2. Few gene expression changes were observed in the SULT family of enzymes, and no overarching trends were apparent; however, changes in specific SULT genes were noted. In particular, SULT1A1 and 4A1 were up-regulated in NASH (not fatty) samples compared to all other groups (normal, steatosis, and NASH fatty), while SULT1C4 expression was increased in both stages of NASH compared to both normal and steatosis. SULT2B1 was elevated in end-stage NASH (not fatty) samples compared to both normal and steatosis. Upon examination of the PAPS-forming enzymes, up-regulation of PAPSS1 was noted in NASH (not fatty) samples compared to steatosis and PAPSS2 was decreased in steatosis compared to normal.

*Protein Levels of Specific UGT and SULT Isoforms in Human NAFLD.* Relative protein levels of UGT1A1, 1A6, 1A9, and 2B10 were determined by immunoblot and densitometric analysis. The densitometric results of all samples in the study are shown in Figure 3 with representative immunoblots depicting four samples from each disease category. No alterations in UGT1A1 protein levels, one of the isoforms responsible for the metabolism of APAP, were observed throughout progression of NAFLD. Additionally, protein levels of UGT2B10, which metabolizes polyunsaturated fatty acids (Bock, 2010), were unaltered during the progression of NAFLD. UGT1A6 protein levels were measured due to its ability to glucuronidate APAP. NASH (fatty) samples exhibited a significant downregulation of UGT1A6 protein compared to steatosis. UGT1A6 protein was also decreased in NASH (not fatty) samples compared to both normal and steatosis. UGT1A9, which also metabolizes APAP, exhibited a significant decrease in protein levels in NASH (not fatty) samples compared to steatosis.

Relative protein levels of SULT1A1, 1C4, and 2A1 were determined by immunoblot and densitometric analysis. The densitometric results of all samples in the study are shown in Figure 4 with representative immunoblots depicting three samples from each disease category. SULT1A1 is the primary isoforms responsible for sulfonation of APAP. SULT1A1 protein was elevated in steatosis compared to normal, but decreased in both stages of NASH compared to steatosis. SULT1C4 has long been considered a primarily fetal protein (Riches et al., 2009;Gamage et al., 2006). Protein levels of SULT1C4 were investigated due to the striking upregulation of SULT1C4 mRNA observed in both stages of NASH (see Figure 2). In the current study, protein levels of SULT1C4 were significantly increased in both stages of NASH compared to both normal and steatosis. Protein levels of SULT2A1 were investigated due to its prominence in drug metabolism and high expression in normal human liver (Riches et al., 2009). Though SULT2A1 protein levels appeared to increase in steatosis, these findings were not statistically significant; however, SULT2A1 protein was significantly decreased in NASH (not fatty) samples compared to steatosis.

*Effect of Human NAFLD on the Glucuronidation and Sulfonation of Acetaminophen (APAP).* UGT activity was determined by reaction of human liver crude membrane preparations with acetaminophen (APAP) and measurement of APAP-glucuronide (APAP-GLUC) by HPLC analysis. The results, expressed as nmol APAP-GLUC formed per minute per mg protein, are shown in Figure 5. No alterations in the ability of UGT enzymes to glucuronidate APAP were observed in any stage of NAFLD. SULT activity was determined by incubation of human liver cytosol preparations with APAP and measurement of APAP-sulfate (APAP-SULF) by HPLC analysis. The results are shown in Figure 5, and expressed as pmol APAP-SULF formed per minute per mg protein. APAP-SULF formation was significantly increased in steatotic samples compared to normal. In contrast, both stages of NASH exhibited a significant decrease in the ability to sulfonate APAP as compared to steatotic samples.

## Discussion

Phase II conjugation enzymes are critically important in the management of several xenobiotics due to their ability to significantly increase the mass and hydrophilicity of a substrate (Zamek-Gliszczynski et al., 2006). The UGTs and SULTs represent two prominent classes of Phase II enzymes as they transform a large proportion of drugs and endogenous molecules including bilirubin, steroid hormones, bile acids, eicosanoids, and neurotransmitters (Jancova et al., 2010;Zamek-Gliszczynski et al., 2006;Bock, 2010;Gamage et al., 2006). Conjugation via UGTs can result in inactivation, formation of pharmacologically active metabolites, or generation of acyl glucuronides with significant potential for toxicity (Zamek-Gliszczynski et al., 2006;Bock, 2010). Cytosolic SULTs are responsible for the metabolism of small, endogenous molecules and drugs, while the carbohydrate and chondroitin SULTs (CHSTs) in the Golgi apparatus are important in maintaining cellular homeostasis through sulfonation of macromolecules including lipids, proteins and glycosaminoglycans (Gamage et al., 2006). Similar to the UGTs, SULT reactions can lead to detoxication as well as bioactivation (Gamage et al., 2006). The SULTs are credited with coordination of high-affinity reactions that dominate at low concentrations, whereas the UGTs prevail at high substrate concentrations (Zamek-Gliszczynski et al., 2006). This is primarily due to the limited stores of the SULT cofactor PAPS, synthesized by PAPSS1 and 2, and largely dependent on the availability of sulfate (Zamek-Gliszczynski et al., 2006). Stores of hepatic UDP-GlcUA which is the cofactor for most UGT enzymes are much higher than that of PAPS, thus allowing for high-capacity metabolism (Meech and Mackenzie, 2010;Mackenzie et al., 2011;Zamek-Gliszczynski et al., 2006). UGT1A1, 1A3, 1A4, 1A6, 1A9, 2B7, and 2B15 and SULT1A1, 1B1, 1E1, and 2A1 are considered most important for drug metabolism; however, perturbations in the expression or function of any isoform can result in

significant consequences not just for drug metabolism but also cellular homeostasis (Riches et al., 2009;Miners et al., 2010).

Liver disease has traditionally been associated with decreased elimination of drugs; however, even in patients with cirrhosis, glucuronidation appears to be maintained while the typical downregulation of CYP activity seen with high levels of inflammation is readily apparent (Debinski et al., 1995). While no studies have investigated the direct effect of human NAFLD progression on UGT expression and function, some studies have indicated a decrease in mRNA of specific isoforms with increasing levels of hepatic inflammation including 1A4, 2B4, and 2B7 (Aitken et al., 2006;Congiu et al., 2002). However, it is known that the mRNA expression of specific UGT isoforms does not always coincide with levels of the subsequent protein due to differential translation efficiency, splicing, or post-translational modifications (Court, 2010). Investigation of human NAFLD progression on SULT expression and function is also lacking. However, studies in hepatocellular carcinoma (HCC) and cirrhosis patients, both of which can arise from NAFLD, have indicated a decreased ratio of APAP-GLUC to APAP-SULF in HCC patients and only a slight decrease in those with cirrhosis which the authors attributed to increased SULT1A1 activity (Wang et al., 2010). Others have utilized a proteomics approach and identified a reduction in SULT1A1 protein in HCC which was verified by immunoblot analysis and measurement of activity using p-nitrophenol (Yeo et al., 2010). The current study indicates an increase in mRNA of specific UGT and SULT isoforms in the more severe, inflammatory stage of NASH, but an overall lack of changes in UGT and SULT mRNA expression at the earlier stage of steatosis.

Currently, we identified an increase in UGT1A9 mRNA in both stages of NASH. UGT1A9 is known for metabolism of bulky phenols while also being infamous for conversion of carboxylic acids to acyl-O-glucuronides, some of which can readily form protein adducts (King et al.,

2000;Ritter, 2000). This enzyme has been shown to conjugate several xenobiotic carboxylic acids and phenols including APAP, naproxen, furosemide, fenoprofen, and ibuprofen (Ritter, 2000;Bock, 2010). Upregulation of UGT1A9 mRNA in NASH creates potential for enhanced formation of acyl-O-glucuronides, but is dependent on subsequent upregulation of protein and activity. UGT1A1, another important enzyme in drug metabolism, is also responsible for metabolism of endogenous molecules such as estradiol, catecholestrogens, polyunsaturated fatty acids, all-trans retinoic acid, and arachidonic acid (Bock, 2010;Ritter, 2000;Miners et al., 2010). Perhaps most importantly, UGT1A1 is the sole enzyme responsible for metabolism of bilirubin, a product of heme degradation, and lack of functional 1A1 protein can result in moderate to severe hyperbilirubinemia (Bock, 2010). Like UGT1A9, 1A1 is able to metabolize APAP as well as buprenorphine and etoposide (Miners et al., 2010;King et al., 2000). We identified an increase in UGT1A1 mRNA above normal, steatosis, and NASH (fatty) samples in the NASH (not fatty/cirrhosis) group, indicating the potential for increased metabolism via 1A1. However, we found very little changes in either UGT1A9 or 1A1 protein levels suggesting little potential for altered glucuronidation mediated by these isoforms. Furthermore, investigation of mRNA levels of UGT1A6, which is also capable of metabolizing APAP, revealed no alterations in transcriptional regulation due to NAFLD progression, but a significant decrease in protein levels in both stages of NASH. In the current study, overall glucuronidation activity toward APAP as assessed at a single concentration and timepoint in crude membrane preparations revealed no significant alterations in the glucuronidation of APAP at any stage of NAFLD. However, the studies by Court et al., identified UGT1A9 as the predominant isoform in the glucuronidation of APAP at clinically relevant concentrations (Court MH et al., 2001). Collectively, the relative protein levels of UGT1A1, 1A6, and particularly,1A9, are congruent with the *ex vivo* enzyme activity assay, further supporting the conclusion that NAFLD progression does not significantly alter the glucuronidation of APAP.

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Investigation of the various SULTs revealed alterations in two unlikely isoforms: 1C4 and 4A1. The SULT1C family has traditionally been considered fetal enzymes, but has recently been shown to be important for estradiol and catecholestrogen metabolism (Lindsay et al., 2008;Hui et al., 2008;Adjei and Weinshilboum, 2002). Moreover, they are believed to be involved in thyroid hormone regulation, and have a role in the metabolism of oral contraceptives (Lindsay et al., 2008;Yasuda et al., 2005). SULT1C4 mRNA and protein levels were elevated in both stages of NASH. However, the transcriptional regulation and pathological importance of SULT1C4 in liver disease are largely unknown, and its role in NASH warrants future investigation. Another interesting and perplexing discovery was the upregulation of SULT4A1 mRNA in NASH (not fatty/cirrhosis). SULT4A1 is almost exclusively expressed in brain, and is very highly conserved amongst species though it has no known endogenous ligand (Gamage et al., 2006;Minchin et al., 2008). One study has shown an elevation in hepatic SULT4A1 expression following ventromedial hypothalamic lesions which are known to cause significant disruptions in metabolic homeostasis manifesting as obesity and hyperinsulinemia similar to that seen in metabolic syndrome, an accompanying feature of NAFLD (Kiba et al., 2009;King, 2006;McCullough, 2011). Expression of hepatic SULT4A1 during metabolic syndrome and currently in NASH is a novel discovery, and its function in the context of liver disease is potentially an exciting new avenue of investigation.

There were only a few critical changes observed in the major drug metabolizing SULTs in the current study. We identified no alteration in SULT2A1 at the transcriptional level, but found a reduction in SULT2A1 protein levels in NASH (not fatty/cirrhosis). In contrast, SULT1A1 mRNA, the most dominant isoform found in the liver (Gamage et al., 2006), was significantly elevated in end-stage, NASH (not fatty/cirrhosis) samples; however, SULT1A1 protein was elevated in steatosis, but decreased in both stages of NASH. The influence of SULT1A1 on the metabolism of APAP cannot be understated and is reflected in the current study. SULT activity in NAFLD

directly mirrored the observed changes in SULT1A1 protein resulting in increased metabolism in steatosis and diminished capacity of APAP sulfonation in NASH. These findings are complementary to our studies in a rodent model of NASH in which the excretion of APAP-SULF into both plasma and bile was decreased, suggesting a reduction in APAP sulfonation (Lickteig et al., 2007). Currently we have identified a reduction in APAP-SULF formation in human NASH samples (see Figure 5) via an *ex vivo* activity analysis of liver cytosols, despite ample supplementation of the cofactor PAPS. This indicates an alteration in the basal function of SULT enzymes, primarily SULT1A1.

This is the first study to investigate and identify alterations in the expression and function of multiple UGT and SULT isoforms in the progressive stages of human NAFLD. Overall, we have found minimal alterations in individual UGTs and their activity during human NAFLD progression; however, several changes in the expression and function of specific SULT enzymes suggest significant perturbations with disease progression.

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**Authorship Contributions**

*Participated in research design:* Hardwick and Cherrington.

*Conducted experiments:* Hardwick, Ferreira, More, and Lake.

*Contributed new reagents or analytical tools:* Slitt and Manautou.

*Performed data analysis:* Hardwick, Ferreira, More, and Lu.

*Wrote or contributed to the writing of the manuscript:* Hardwick, Ferreira, More, Manautou, Slitt, and Cherrington.

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## Footnotes

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**Figure Legends**

*Figure 1. Log-Transformed Relative mRNA Expression of UGT Isoforms in Human NAFLD.* Relative mRNA levels of UGT1A1, 1A3, 1A4, 1A6, 1A9, 2A3, 2B4, 2B7, 2B10, 2B15, 2B17, 2B28, 3A1, and 3A2 in human liver samples diagnosed as normal, steatotic, NASH (fatty), and NASH (not fatty) are shown. Total RNA was isolated and measured by the Quantigene 2.0 Assay for RNA analysis. Data were normalized to GAPDH and presented as log transformed relative mRNA expression. Asterisk (*) indicates significant difference from normal and dagger (†) indicates significance from steatosis ($p \leq 0.05$).

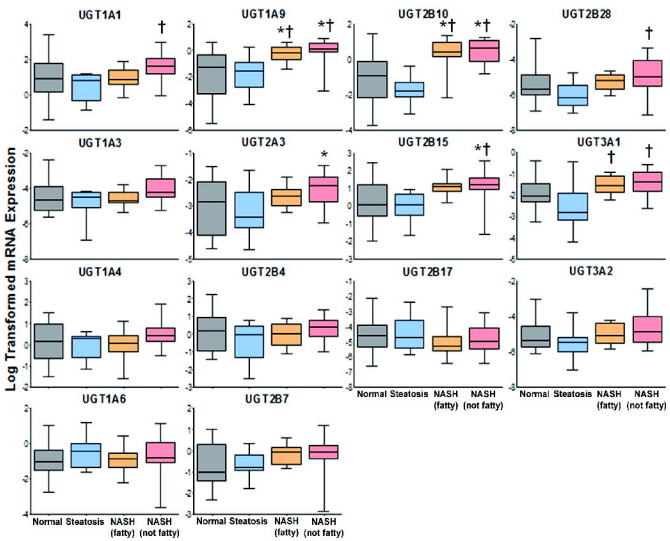
*Figure 2. Log-Transformed Relative mRNA Expression of SULT Isoforms in Human NAFLD.* Relative mRNA levels of SULT1A1, 1A2, 1B1, 1C2, 1C4, 1D1P, 1E1, 2A1, 2B1, 4A1, and PAPSS1 and PAPSS2 in human liver samples diagnosed as normal, steatotic, NASH (fatty), and NASH (not fatty) are shown. Total RNA was isolated and measured by the Quantigene 2.0 Assay for RNA analysis. Data were normalized to GAPDH and presented as log transformed relative mRNA expression. Asterisk (*) indicates a significant difference from normal, dagger (†) indicates significance from steatosis, and double dagger (‡) indicates significance from NASH (fatty) with a significance level of $p \leq 0.05$.

*Figure 3. Relative Protein Levels of UGT1A1, 1A6, 1A9, and 2B10 in Human NAFLD.* Representative immunoblots of hepatic UGT1A1, 1A6, 1A9, and 2B10 protein levels are shown with pan-Cadherin as control and 4 samples from each diagnostic category as follows: normal, steatosis, NASH (fatty), and NASH (not fatty). Relative protein levels of all samples in the study were determined by densitometric analysis and expressed as relative to pan-Cadherin. Asterisk (*) indicates a significant difference from normal and dagger (†) indicates significance from steatosis ($p \leq 0.05$).

*Figure 4. Relative Protein Levels of SULT1A1, 1C4, and 2A1 in Human NAFLD.* Representative immunoblots of hepatic SULT1A1, 1C4, and 2A1 protein levels are shown with Total ERK as control and 4 samples from each diagnostic category as follows: normal, steatosis, NASH (fatty), and NASH (not fatty). Relative protein levels of all samples in the study were determined by densitometric analysis and expressed as relative to Total ERK. Asterisk (*) indicates a significant difference from normal and dagger (†) indicates significance from steatosis ($p \leq 0.05$).

*Figure 5. Enzymatic Activity of Multiple UGT and SULT Isoforms Against the Substrate, Acetaminophen (APAP).* Activity of pan-UGT isoforms was performed in human liver microsomes against APAP and expressed as nmol APAP-GLUC/min/mg protein. Activity of multiple SULT isoforms was determined in human liver cytosol preparations against APAP and expressed as pmol APAP-SULF/min/mg protein. Asterisk (*) indicates a significant difference from normal and dagger (†) indicates a significant difference from steatosis ($p \leq 0.05$).

Figure 1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UGT1A9
UGT2B10
UGT2B28
UGT1A3
UGT2A3
UGT2B15
UGT3A1
UGT1A4
UGT2B4
UGT2B17
UGT3A2
UGT1A6
UGT2B7
Log Transformed mRNA Expression
Normal
Steatosis
NASH (fatty)
NASH (not fatty)

Figure 2

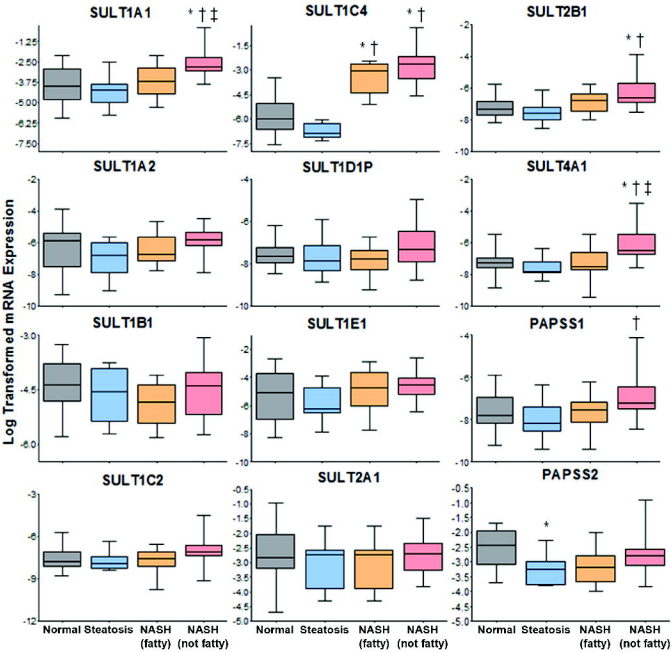

SULT1A1
SULT1C4
SULT2B1
SULT1A2
SULT1D1P
SULT4A1
SULT1B1
SULT1E1
PAPSS1
SULT1C2
SULT2A1
PAPSS2
Log Transformed mRNA Expression
Normal
Steatosis
NASH (fatty)
NASH (not fatty)

Figure 3

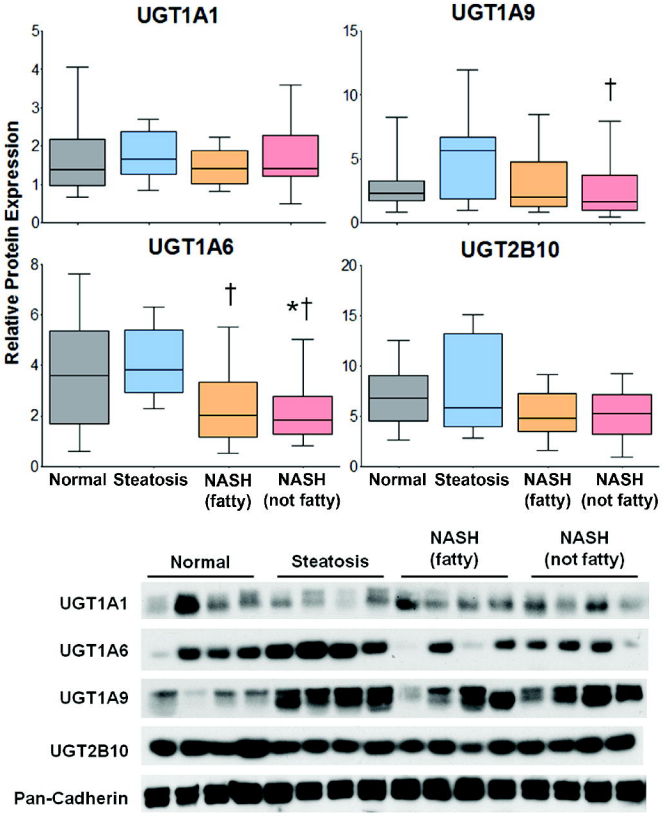

UGT1A1
UGT1A9
UGT1A6
UGT2B10
Relative Protein Expression
†
*†
Normal
Steatosis
NASH (fatty)
NASH (not fatty)
Pan-Cadherin

Figure 4

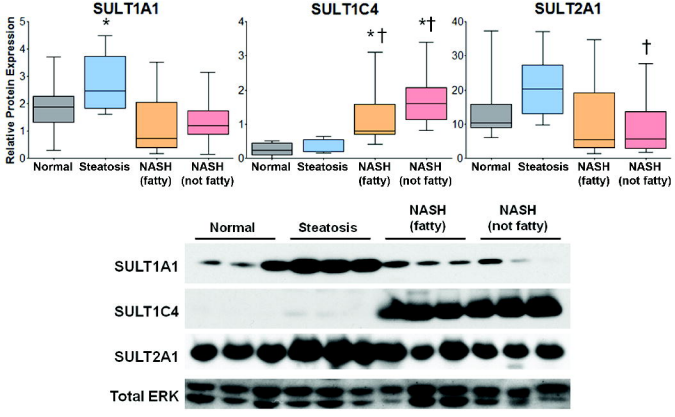
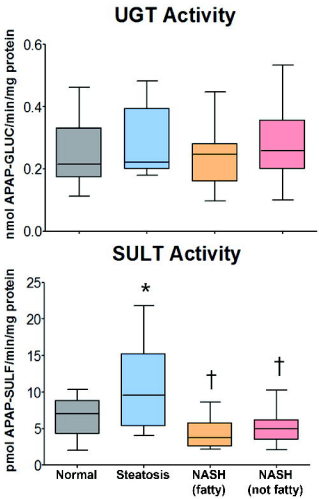

SULT1A1
SULT1C4
SULT2A1
Relative Protein Expression
Normal
Steatosis
NASH (fatty)
NASH (not fatty)
Normal
Steatosis
NASH (fatty)
NASH (not fatty)
SULT1A1
SULT1C4
SULT2A1
Total ERK

Figure 5


UGT Activity
nmol APAP-GLUC/min/mg protein
0.6
0.4
0.2
0.0
SULT Activity
pmol APAP-SULF/min/mg protein
25
20
15
10
5
0
*
†
†
Normal
Steatosis
NASH (fatty)
NASH (not fatty)