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Alcohol Cirrhosis Alters Nuclear Receptor and Drug Transporter Expression in Human Liver

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Abbreviations: ABC, ATP binding cassette family; FXR, Farnesoid-X-receptor; GPX, Glutathione Peroxidase; PXR, Pregnane-X-receptor; Nrf2, Nuclear factor E2 related factor 2; SLC, Solute carrier; CYP, Cytochrome P450; NQO1, NADPH quinone oxidoredictase 1; ST, Sucrose Tris; HPRT1, Hypoxanthine phosphoribosyl transferase 1; PBS/T, Phosphate-buffered saline with tween 20; Shp, Small heterodimer protein

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Abstract

Unsafe use of alcohol results in approximately 2.5 million deaths worldwide, with cirrhosis contributing to 16.6% of reported deaths. Serum insulin levels are often elevated in alcoholism, which may result in diabetes; which is why alcoholic liver disease and diabetes often are co-present. Because there is a sizable population that presents with these diseases alone or in combination, the purpose of this study was to determine whether transporter expression in human liver is affected with alcoholic cirrhosis, diabetes, and alcohol cirrhosis co-existing with diabetes. Transporters aid in hepatobiliary excretion of many drugs and toxic chemicals, and can be determinants of drug-induced liver injury. Drug transporter and transcription factor relative mRNA and protein expression in normal, diabetic, cirrhotic and cirrhosis with diabetes human livers were quantified. Cirrhosis significantly increased ABCC4, 5, ABCG2 and SLCO2B1 mRNA expression, and decreased SLCO1B3 mRNA expression in liver. ABCC1, 3-5, ABCG2 protein expression was also upregulated by alcohol cirrhosis. ABCC3-5, and ABCG2 protein expression was also upregulated in diabetic-cirrhosis. Cirrhosis increased NRF2 mRNA expression, whereas it decreased PXR and FXR mRNA expression in comparison to normal livers. Hierarchical cluster analysis indicated that expressions of ABCC2, 3 and 6; SLCO1B1 and 1B3; and ABCC4 and 5 were more closely related in the livers from this cohort. Overall, alcohol cirrhosis altered transporter expression in human liver.

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Introduction

Hepatobiliary excretion is an integral function necessary to excrete bile acids, bilirubin, conjugated hormones, as well as, drugs and chemicals from liver (Klaassen and Aleksunes, 2010). The process of biliary excretion relies upon membrane bound transporters localized to hepatocytes, which extract chemicals from blood and efflux chemicals into bile. The solute carrier organic anion (SLCO) and ATP-Binding Cassette (ABC) transporter families comprise two major families that mediate hepatic uptake and efflux processes.

SLCO transporters are often described as “uptake transporters”, because they are predominantly localized to the sinusoidal membrane and typically extract chemicals from blood into hepatocytes (reviewed by (Klaassen and Aleksunes, 2010)). In humans, SLCO1B1, 1B3, 2B1 and 1A2 have relatively high expression in liver. SLCO1B1, 1B3, and 2B1 transport a diverse range of drugs including benzylpenicillin, statins, and estradiol glucuronide (Klaassen and Aleksunes, 2010). Identification of SNPs in the *SLCO1B1* gene and resulting SLCO1B1 polymorphisms results cause altered disposition of statins (Generaux et al., 2011). Human SLCO mRNA expression is regulated through transcription factor-mediated pathways, such as Liver-X-Receptor (LXR), Farnesoid-X-Receptor (FXR), Constitutive Androstane Receptor (CAR), Pregnane-X-Receptor (PXR) (Svoboda et al., 2011).

The ATP-binding cassette (ABC) transporter superfamily facilitates chemical efflux; and includes Multidrug Resistance Proteins (ABCB), Multidrug Resistance-Associated

Proteins (ABCC), Bile Salt-Export Pump (ABCB11), and Breast Cancer Resistance Protein (ABCG2). In liver, ABCC2, ABCG2 and ABCBs are localized to the canalicular membrane and facilitate biliary excretion of chemicals. ABCC1, 3-6 are localized sinusoidally and/or basolaterally, and efflux chemicals from hepatocytes into blood. Similar to SLCOs, human ABCC expression is modulated by transcription factors, such as Nuclear Factor-E2 related factor 2 (NRF2), CAR, PXR, and FXR (Klaassen and Slitt, 2005).

Alterations in transporter expression and function due to hepatic stress have been noted and can have significant implications on the fate of numerous drugs. Hepatic steatosis resulting from obesity and/or diabetes resulted in significant alterations in transporter expression in hepatocytes, as demonstrated in mouse models (Cheng et al., 2008; More and Slitt, 2011; More et al., 2012). As compared to steatosis, cirrhosis is a significant hepatic stress with replacement of normal functional tissue by scar tissue, which is unable to maintain the functions of the liver. According to Center for Disease Control and prevention (CDC), more than 15,000 Americans die every year from alcoholic liver cirrhosis (National Vital Statistics Report, Volume 60, No 3). Other major causes of cirrhosis include chronic viral hepatitis, non-alcoholic steatohepatitis (NASH), and damaged or blocked bile flow (Anand, 1999). About 30% of cirrhotic patients also suffer from diabetes (Hickman and Macdonald, 2007). Acute, as well as chronic alcohol consumption leads to development of insulin resistance, which can progress to diabetes mellitus (Kim and Kim, 2012). Disruption of normal functions of the liver in cirrhosis may lead to hepatogenous diabetes (Garcia-Compean et al., 2009). Additionally, obesity

and diabetes mellitus increase the severity of alcoholic liver disease (Raynard et al., 2002). Owing to interplay between diabetes and cirrhosis, the two conditions often co-present clinically (Baig et al., 2001).

Since many human Phase-I and -II biotransformation enzymes are coordinately regulated by transcription factors that regulate transporter expression, representative cytochrome p450 (CYP), UDP glucuronosyl transferase (UGT), and Nad(p)h:quinone oxidoreductase (NQO1) mRNA expression was also determined. The purpose of this study was to determine whether alcohol cirrhosis alone, or in combination with diabetes, alter transporter expression in intact human liver, as transporters are integral for the hepatobiliary clearance of drugs, bile acids, and bilirubin. Our study has included analysis of livers from subjects who presented with steatosis or diabetes without cirrhosis, as these diseases are sometimes present in alcoholics. Our findings herein illustrate coordinated alterations in the expression of certain SLCO and ABC transporter members in human alcohol cirrhotic liver tissues.

Materials and Methods

Human liver tissues. Liver tissues from normal healthy, alcohol cirrhotic, steatotic, and diabetic-cirrhotic (co-existence of alcohol cirrhosis and diabetes) subjects were obtained from Liver Tissue Cell Distribution System (LTCDS), University of Minnesota (Minneapolis, MN). Additional liver lysates in Trizol reagent from normal and diabetic subjects were purchased from Xenotech LLC (Lenexa, KS) and were only analyzed for mRNA expression. The details of subject age, gender and ethnicity are mentioned in supplementary material (Supplementary table S1). Exemption approval from the University of Rhode Island Institutional Review Board was granted before tissues were procured.

RNA Extraction. Total RNA from liver was isolated by phenol-chloroform extraction using RNA Bee (Tel-Test Inc, Friendswood, TX) according to the manufacturer's protocol. Tissue lysates obtained in Trizol were directly homogenized and subject to chloroform extraction. RNA concentration was quantified by absorbance at 260 nm (Nanodrop ND1000, Thermo Fisher Scientific, Waltham, MA). Agarose gel electrophoresis followed by UV illumination was used to visualize RNA and confirm integrity.

Quantigene Plex 2.0 assay for mRNA quantification. Only samples in which total RNA looked intact and not degraded were subjected to analysis the QuantiGene Plex 2.0 assay (Affymetrix, Santa Clara, CA). However, a benefit to this technology according to the manufacturer is that it allows for detection of partially degraded mRNA

transcripts, which is desirable for RNA isolated from human tissue. The protocol for the assay is described elsewhere (Aleksunes et al., 2009). Briefly, 1.1 µg total RNA was incubated with beads with capture probe, label extender and blocker. On day two, the beads were washed and incubated with amplifier, and subsequently with label. Then incubation with streptavidin containing substrate was used for detection on BioPlex Luminometer.

Tissue fractionation. Approximately 100mg of tissue was homogenized in Sucrose-Tris (ST) buffer (250 mM sucrose, 10 mM Tris-HCl buffer, pH 7.4) and containing protease inhibitor cocktail (2 µg/mL, Sigma-Aldrich, Co, St. Louis, MO). Homogenates were centrifuged at 100,000 xg for 60min at 4°C. The resulting pellet is a typical fraction used to detect transporter expression as described by our previous publications as well as multiple other research groups (Trauner et al., 1997; Aleksunes et al., 2006; Campion et al., 2008; Cheng et al., 2008; Maher et al., 2008). The supernatant was saved as a cytosolic fraction to measure NQO1 and GPX1 protein expression. ST buffer (200 µl) was used to re-suspend the resulting pellet. Nuclear fractions from approximately 100 mg of liver tissue were isolated using a NE-PER nuclear extraction kit (Thermo Scientific, Rockford, IL) according to the manufacturer's instructions. Protein concentration of the membrane fractions was determined using the DC protein assay (Bio-Rad Laboratories, Hercules, CA).

Western blot analysis. Western blots were used to quantify the relative expression of transport proteins in human liver tissues, as described in our previous publication (More

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and Slitt, 2011; More et al., 2012). Briefly, the membrane/ nuclear extracts were separated on polyacrylamide gel (10% resolving, 4% stacking), transblotted on PVDF membrane, and blocked with 2% non-fat dry milk in phosphate buffered saline with Tween 20 (PBS/T). The membranes were then incubated with specific primary and secondary antibody, and then with ECL+ fluorescence reagent. The blots were then developed on X-ray films; protein bands on the resulting autoradiographs were quantified using Quantity One® software v4.6.3 (Biorad, Hercules, CA). Supplementary material (Supplementary table S-2) provides the antibody source and western blot conditions. OATP1B1 and 1B3 protein expression by Western blot was not determined due to lack of high quality commercially available antibodies.

Statistical analysis. Raw data from mRNA quantification was normalized to housekeeping gene hypoxanthine phosphoribosyl transferase 1 (HPRT1). Log transformed normalized data was more approximately normally distributed as compared with non-transformed data. Within each gene, pairwise comparison of expression between disease groups was tested a one-way ANOVA followed by a Tukey Honestly Significant Difference (HSD) test. Data from protein quantification was plotted as percent expression and analyzed by one-way ANOVA followed by Dunnett's *post hoc* test. Difference of $p \leq 0.05$ was considered statistically significant. Asterisks (*) represent a statistical difference ($p \leq 0.05$) from normal non-steatotic livers, and dots (●) represent outliers. Hierarchical clustering analysis with Pearson correlation as a similarity measurement was also done to discover potential groups of genes with high correlation.

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Results

Transporter mRNA expression in liver is altered by alcohol cirrhosis and diabetic-cirrhosis. Alcohol cirrhosis altered mRNA expression of some transporters (Fig. 1A). SLCO1B1 mRNA expression was similar among all groups examined. SLCO1B3 mRNA expression was significantly decreased in livers from alcohol cirrhosis patients compared to normal non-steatotic livers. In contrast, SLCO2B1 mRNA expression was increased with alcohol cirrhosis compared to normal non-steatotic livers.

In liver, ABCC transporters are localized to the canalicular (ABCC2 and ABCG2) or sinusoidal membranes (ABCC1, 3-6) of hepatocytes, and mediate organic anion efflux from hepatocytes (Klaassen and Aleksunes, 2010). ABCC1, 4 and 5 mRNA expression was increased in alcohol cirrhotic livers compared to normal non-steatotic livers (Fig. 1B). ABCC2 mRNA expression remained unchanged between the groups compared, whereas ABCG2 expression was increased in livers from subjects with alcohol cirrhosis (Fig. 1B). Diabetic-cirrhosis decreased ABCC3 expression compared to normal non-steatotic livers. ABCC6 mRNA expression was similar among from normal, steatotic, alcohol cirrhotic, diabetic-cirrhotic, and diabetic livers.

Correlation analysis was performed to examine transporter expression that is regulated similarly. Table 1 illustrates the correlation among mRNA expression of above-mentioned transporters in the entire sample set. SLCO1B1 expression correlated with SLCO1B3, 2B1, ABCC2-4, and ABCC6 expression. Similarly, SLCO1B3 mRNA expression correlated with ABCC2-6 mRNA expression. SLCO2B1 expression also correlated with all ABC transporters analyzed in this study. ABCC2 mRNA expression was correlated with ABCC3, 4, 6 and ABCG2 mRNA expression. ABCC3 mRNA

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expression was also correlated with ABCC6 and ABCG2 mRNA expression. ABCC4 expression was correlated with ABCC5 and ABCG2 expression. Correlation was also observed between ABCC6 and ABCG2 expression.

Transporter protein expression is altered in livers from subjects with steatosis, alcohol cirrhosis, and diabetic-cirrhosis. Fig. 2 illustrates the effect of steatosis, alcoholic cirrhosis, and diabetic-cirrhosis on transporter protein expression in fractions from intact human liver tissue (representative blots). Alcoholic cirrhosis and diabetic-cirrhosis increased ABCC1, 3, and 5 protein expression compared to normal non-steatotic livers. ABCC2 protein remained unchanged between all the groups. ABCC4 and ABCG2 protein expression was increased in livers with steatosis, alcohol cirrhosis and diabetic cirrhosis. In contrast to other ABC transporters, ABCC6 protein expression decreased in livers with alcohol cirrhosis and diabetic cirrhosis.

Alcoholic cirrhosis and diabetic-cirrhosis affect transcription factor expression in intact human liver. Studies in recent years have revealed several transcription factor-mediated pathways (e.g. PXR, CAR, and FXR), as well as the antioxidant response (e.g. NRF2), are important mediators of SLCO and ABC transporter regulation in liver (Klaassen and Aleksunes, 2010). Therefore, NRF2, PXR, CAR, and FXR expression was also evaluated and correlated with transporter expression. Fig. 3A depicts the PXR, CAR FXR, and NRF2 mRNA expression in human liver. NRF2 mRNA expression was increased in alcohol cirrhotic and diabetic-cirrhotic livers compared to normal non-steatotic livers. PXR mRNA expression was decreased in livers with diabetic-cirrhosis,

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as compared to normal livers. CAR mRNA expression remained unchanged between all groups analyzed. FXR mRNA expression was decreased in livers with alcohol cirrhosis and diabetic-cirrhosis.

Table 2 illustrates the correlation among abovementioned transcription factor mRNA expressions. CAR mRNA expression correlated significantly with FXR and PXR mRNA expression; and PXR expression correlated with FXR expression.

Alcohol cirrhosis affects phase-I and phase-II drug metabolizing enzymes mRNA expression. Correspondingly, Figure 3B depicts mRNA expression for representative CYP and UGTs, along with FXR target gene, Small Heterodimer Protein (SHP). UGT1A3 mRNA expression was increased in steatotic livers compared to normal livers. CYP3A4 mRNA expression was increased in livers with steatosis, but similar to normal livers in the other disease conditions. CYP2B6 mRNA expression was decreased in livers with diabetic-cirrhosis compared to normal non-steatotic livers. SHP mRNA expression was similar among all the disease conditions tested in the study. Other CYP and UGT isoforms including CYP2D6, UGT1A1, 1A4 mRNA expressions were also studied, and remained unchanged between the groups (data not shown).

Alcohol cirrhosis increases NRF2, NQO1, and Glutathione Peroxidase protein expression. NRF2 protein expression in liver fractions was correspondingly increased in alcohol cirrhotic and diabetic-cirrhotic livers compared to normal non-steatotic livers (Fig. 3C and 3D). NQO1 and Glutathione Peroxidase 1 (GPX1), enzymes, which are regulated via NRF2, were also quantified at protein level. NQO1 protein expression

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was increased in steatotic, alcohol cirrhotic and diabetic-cirrhotic livers compared to normal livers, with the most prominent increase present in alcohol cirrhosis. GPX1 protein expression was increased in liver fractions from subjects with alcohol cirrhosis and diabetic-cirrhosis.

Alcohol cirrhosis increases inflammatory cytokine mRNA expression. Fig. 4 demonstrates mRNA expression of inflammatory cytokines tumor necrosis factor α (TNFα), and interleukin 1β (IL1β) in livers. TNFα mRNA expression was increased in both steatosis and alcohol cirrhosis groups, as compared to normal non-steatotic livers. IL1β expression was increased only with steatosis as compared to normal livers.

Hierarchical cluster analysis of transporter and transcription factor mRNA expression. Fig. 5 depicts the correlations between transcription factor and transporter mRNA expression. ABCG2 and SLCO2B1 expression were closely related to CAR expression. Similarly, expression of ABCC4, ABCC5 and NRF2 were closely related. Expression of ABCC2 and PXR were also closely related, and more distantly related to SLCO1B3 and 1B1 expression.

Discussion

This study demonstrated predominant increased mRNA and protein of efflux transporters, such as ABCG2, ABCC1, 3-5 in intact livers of human subjects with alcohol cirrhosis. Uptake transporter expression was less consistent, with decreased SLCO1B3 and increased SLCO2B1 mRNA expression occurring in livers with alcoholic cirrhosis. Transcription factors that regulate transporter expression were also correspondingly altered. NRF2 mRNA and protein expression was increased in alcoholic cirrhotic livers, whereas FXR mRNA expression was decreased.

Hierarchical cluster analysis of transcription factors and transporters obtained in this study is in agreement with the findings in literature. In rodents as well as in humans, NRF2 is known to regulate expression of efflux transporters ABCC2-5 (Klaassen and Slitt, 2005). In the cluster analysis in the present study, ABCC4 and 5 were expressed together with NRF2. Similarly, SLCO2B1 and CAR were expressed together, as observed in rodents (Cheng et al., 2005). ABCC2 and PXR were also clustered together, as also described (Klaassen and Slitt, 2005). SLCO1B1 and 1B3 are reported to be regulated by same transcription factors hepatocyte nuclear factor (HNF) 1α, aryl hydrocarbon receptor (AHR) and CAR (Klaassen and Aleksunes, 2010), and were clustered together in present data. CAR and PXR activation have been shown to increase ABCC2 and 3 expression in hepatocytes (Teng and Piquette-Miller, 2005), indicating that these two transporters also have significant correlation in expression. CAR is known to regulate ABCC2, 3 as well as SLCO1B1, indicating significant correlation in the expression of these three transporters.

Transporter expression in human livers with alcohol cirrhosis has not been characterized comprehensively before this study. Rodent models for alcohol-induced liver disease display steatosis and some degree of fibrosis, but no model fully progresses to the human level of cirrhotic liver (Lieber et al., 1965; Tsukamoto et al., 1986). Previous studies with hepatic transporter expression are with small sample size and/or different liver pathologies like hepatitis C, hepatocellular carcinoma, NASH, or from non-diseased human livers (Nishimura and Naito, 2005; Hilgendorf et al., 2007; Ogasawara et al., 2010; Doi et al., 2011). Hepatitis C virus (HCV) -related cirrhosis increased ABCC4 mRNA and protein expression and ABCC1 mRNA expression in human livers (Ogasawara et al., 2010). It was also noted that ABCG2, SLCO1B1, and 1B3 mRNA expression was decreased in HCV related cirrhosis. Findings of present study for ABCC1, 4, SLCO1B3 are similar to that observed with HCV-related cirrhosis, but ABCG2 and SLCO1B1 differed. Efflux transporter expression in human livers with primary biliary cirrhosis (PBC) was also similar to what was observed in alcohol cirrhotic livers (Zollner et al., 2003). ABCC3 protein expression was increased in PBC and alcohol cirrhosis. The uptake transporter, SLCO1B1, however, remained unchanged with alcohol cirrhosis, but went down with PBC (Zollner et al., 2003). Fatty and non-fatty NASH also enhanced the mRNA and protein expression of ABCC1, 4 and 5 in human livers (Hardwick et al., 2011). These comparisons of the present study with existing findings suggest that these alterations in transporter expression are likely a general response to cirrhosis of any etiology.

Another study reported a patient having lowered SLCO1B3 expression in hepatocellular carcinoma nodule (Doi et al., 2011), which is consistent with the present data that

illustrate decreased SLCO1B3 mRNA expression in alcohol cirrhosis too. As other models of liver injury (e.g. acetaminophen, carbon tetrachloride, cholestasis) also increase efflux transporter expression, we acknowledge that the observation was anticipated. However, because alcohol cirrhosis plagues about 20% of the alcoholic people worldwide, knowing whether aberrant transporter and nuclear receptor expression is present in liver is of toxicological significance because it can provide mechanistic understanding of drug-induced liver injury or altered drug efficacy in patients with alcoholic liver disease.

Transporters facilitate absorption, distribution and elimination of xenobiotics, as well as endobiotics such as bile acids, cholesterol, and conjugated hormones (e.g. estrogens and thyroid hormones) (Klaassen and Aleksunes, 2010). Alterations in the transporter expression or polymorphisms have been associated with alterations in disposition and adverse effects/ protection against adverse effects of certain xenobiotics. Simvastatin-induced myopathy, which is concentration dependent side effect, was found associated with SLCO1B1 polymorphism in human subjects (reviewed by (Niemi et al., 2011)). In another study with methotrexate, it was observed that variants of SLCO1B1 were associated with increased clearance and gastrointestinal toxicity as a side effect in children with acute lymphoblastic leukemia (Trevino et al., 2009). In a different study, mice with increased Abcc3 and 4 expression in liver had enhanced metabolite excretion and were protected against acetaminophen induced hepatocyte injury (Slitt et al., 2003; Aleksunes et al., 2008) and mice lacking Abcc2, 3 and Abcg2 demonstrate mild hepatotoxicity when administered diclofenac (Lagas et al., 2010). The present study

illustrates that intact livers from subjects with alcohol cirrhosis have alterations in major drug transporter mRNA and protein expression in liver. As transporters play a vital role in drug disposition, the findings in this study imply that subjects with above mentioned disease conditions need a consideration while administering drugs that form glucuronide, which are pharmacologically active.

With progression of NAFLD, the expression of Nrf2 and its target genes increases, as determined by immunohistochemistry in human livers (Hardwick et al., 2010). Alcohol-induced oxidative stress also activates Nrf2 in human hepatocytes (Nussler et al., 2010). Alcohol induces lipid deposition in liver; and metabolism of fatty acids as well as ethanol causes generation of oxidative stress in liver (Syn et al., 2009). Alcohol cirrhotic livers in the current study also displayed increased Nrf2 protein levels in nuclear fractions, which is likely a response to increased oxidative stress in the alcoholic liver. PXR is indicated in therapeutic applications against inflammatory liver diseases. PXR activation by pregnenolone-16-alpha carbonitrile leads to decreased carbon tetrachloride induced fibrogenesis in rats (Marek et al., 2005). The decreased PXR expression may be an indicator that PXR deficiency correlates with increased risk for liver disease. FXR regulates bile acid homeostasis, TG and cholesterol metabolism, glucose homeostasis and fibrogenesis in liver (reviewed by (Fuchs, 2012)). FXR activation by bile acids induces PPARα expression, and this increases β-oxidation of fatty acids (Pineda Torra et al., 2003). Thus FXR activation may protect liver from fat deposition in both alcoholic, as well as, non-alcoholic liver diseases. In present study, FXR mRNA expression was decreased in alcohol cirrhosis, suggesting that FXR suppression might occur during alcoholic liver disease, which could be a mechanism for

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alcoholic liver injury. Inflammation could be a possible factor contributing to the alterations in nuclear receptors analyzed in this study. Lipopolysaccharide treatment of mice resulted in decreased PXR signaling and target gene expression in mice (Moriya et al., 2012). Similarly, treatment of Huh7 cells with inflammatory cytokines TNFα and IL6 resulted in marked decrease in FXR target transporter BSEP (Chen et al., 2012). As disease progression of cirrhosis involves increase in inflammation, decreased mRNA expression of PXR/ FXR in alcohol cirrhosis/ diabetic cirrhosis could possibly be explained. Further studies are necessary in order to elucidate why PXR and FXR expression is decreased in alcohol cirrhosis, and whether the decreased expression contributes to the development of alcohol cirrhosis.

Elbekai et. al., in 2004 reported that certain Phase-I biotransformation enzyme expression was altered expression in livers of cirrhotic subjects. CYP1A and CYP3A showed reduced expression with cirrhosis, whereas CYP2C, 2A and 2B remained unaltered (Elbekai et al., 2004). The present data display little or no change in CYP isoform mRNA expression. Similarly, glucuronidation activity in liver is reported to be unaltered with cirrhosis (Elbekai et al., 2004). The present study had results consistent with this observation – UGT1A1, 1A3, 1A4, and 2B7 expression was remained unchanged between normal and alcohol cirrhotic livers, although it should be noted that UGT1A3 was decreased in diabetic-cirrhosis livers and UGT2B7 was decreased in diabetic livers.

In summary, we demonstrate that alcohol cirrhosis significantly alters transporter expression in human liver, most notably altering ABCC3, ABCC4, and, ABCC5, which was associated with altered NRF2, CAR, and FXR mRNA expression. In this study,

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Diabetes did not significantly alter mRNA expression of the transporters analyzed. However, as this is a small sample set, and expression was quantified only on the mRNA level, further studies will be needed to comprehensively address the effect of diabetes on transporters. Additionally, the presence of diabetes in combination with cirrhosis did not augment the effect of cirrhosis on transporter expression. Significant correlations between transporter and nuclear receptor expression were observed in the cohort of livers analyzed. Overall, the data herein illustrate alterations in hepatic transporter expression in the alcohol cirrhotic liver that correlates to changes in nuclear receptor expression. Alterations in nuclear receptor and drug transporter expression in alcoholic liver should be given consideration when evaluating altered drug toxicities.

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Authorship contributions

Participated in research design: More, Slitt, Cherrington, Cheng, Buckley

Conducted experiments: More, Cheng, Donepudi

Contributed new reagents or analytic tools: Buckley

Performed data analysis: More, Slitt, Lu

Wrote or contributed to the writing of the manuscript: More, Slitt, Cherrington, Lu, Buckley

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Footnotes

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Figure Legends

Fig. 1. Transporter mRNA expression in livers from normal, steatotic, alcohol cirrhotic, diabetic-cirrhosis, and diabetic subjects. (A) SLCO1B1, 1B3 and 2B1 mRNA expression, (B) ABCC1-6, and ABCG2 mRNA expression. Total RNA was isolated from intact human donor liver tissue (Normal, n=22; Steatosis, n=8; Alcohol cirrhosis, n=19; Diabetes, n=20; Diabetic-cirrhosis, n=9) and relative mRNA expression was quantified. Raw data was normalized to HPRT1 and log transformed before comparison. Asterisks (*) represent a statistical difference ($p \leq 0.05$) from normal non-steatotic livers and dots (●) represent outliers. SLCO1B3 mRNA expression was decreased, whereas 2B1 was increased in alcohol cirrhosis compared to normal non-steatotic livers. ABCG2, ABCC4 and 5 mRNA expression was increased in livers from donors with alcohol cirrhosis compared to normal non-steatotic livers. ABCC3 expression decreased in diabetic-cirrhosis livers.

Fig. 2. Protein expression of transporters in livers from normal, steatotic, alcohol cirrhotic, and diabetic-cirrhosis subjects by western blot. (A) Relative ABCC1-6, and ABCG2 protein expression was determined in fractions isolated from intact human liver by Western blot. Lanes 1-5 (normal, non-steatotic), 6-10 (steatosis), 11-20 (alcohol cirrhosis), and 21-25 (diabetic-cirrhosis) represent samples analyzed. (B) Quantification of western blots. Protein bands were quantified using Quantity One® software v4.6.3 (Biorad, Hercules, CA). Asterisks (*) represent a statistical difference ($p \leq 0.05$) from normal non-steatotic livers and dots (●) represent outliers. Steatosis increased ABCC4 and ABCG2 protein expression compared to normal livers. ABCC1, 3, 5 protein

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expression was increased, whereas ABCC6 was decreased in alcohol cirrhotic and diabetic-cirrhotic livers as compared to normal non-steatotic livers. ABCC4 and ABCG2 expression was increased in livers with steatosis, alcohol cirrhosis and diabetic-cirrhosis.

Fig. 3. Transcription factor and biotransformation enzyme expression in livers from normal, steatotic, alcohol cirrhotic, diabetic-cirrhotic, and diabetic subjects. (A) Relative PXR, CAR, FXR and NRF2 mRNA expression in human liver. Total RNA was isolated from intact human donor liver tissue (Normal, n=22; Steatosis, n=8; Alcohol cirrhosis, n=19; Diabetes, n=20; Diabetic-cirrhosis, n=9) and relative mRNA expression was quantified. Alcohol cirrhosis and diabetic cirrhosis increased NRF2, but decreased FXR mRNA levels. Diabetic-cirrhosis also decreased PXR mRNA levels. (B) CYP3A4, 2B6, UGT1A3, and 2B7 mRNA expression in liver. Total RNA was isolated from intact human donor liver tissue (Normal, n=22; Steatosis, n=8; Alcohol cirrhosis, n=19; Diabetes, n=20; Diabetic-cirrhosis, n=9). Steatosis increased CYP3A4 and UGT1A3 mRNA expression as compared to normal non-steatotic livers. CYP2B6 mRNA expression was decreased in diabetic livers as compared to normal non-steatotic livers. (C) Relative NRF2, NQO1, and GPX1 protein expression in nuclear (NRF2) and cytosolic (NQO1, GPX1) fractions in livers of normal, steatotic, cirrhotic and diabetic-cirrhotic subjects. Lanes 1-5 (normal, non-steatotic), 6-9 (steatosis), 10-16 (alcohol cirrhosis), and 17-21 (diabetic-cirrhosis) represent samples analyzed. (D) Quantification of NRF2, NQO1, and GPX1 western blots. Asterisks (*) represent a statistical difference ($p \leq 0.05$) from normal non-steatotic livers and dots (●) represent outliers. Nuclear NRF2 (approximately 110 kDa) and cytosolic NQO1, GPX1 protein (31 kDa and

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23 kDa) levels were increased in livers of alcohol cirrhosis and diabetic-cirrhosis subjects as compared to normal livers. NQO1 was also increased in steatotic livers.

Fig. 4. Inflammatory cytokine mRNA expression in livers from normal, steatotic, alcohol cirrhotic, diabetic-cirrhotic, and diabetic subjects. Inflammatory cytokine tumor necrosis factor α (TNFα) and interleukin 1β (IL1β) mRNA expression. Steatosis increased mRNA expression of both TNFα and IL1β, and alcohol cirrhosis increased expression of only TNFα, as compared to normal non-steatotic livers.

Fig. 5. Hierarchical cluster analysis of different transporters and transcription factors. Target gene expression was normalized to HPRT1 and log transformed to use for cluster analysis. Cluster analysis was performed by using squared Pearson's correlation (R^2) as a similarity measure. Genes were clustered as a group with bigger R^2. ABCG2 and SLCO2B1 expression were closely related to CAR expression. Similarly, expression of ABCC4, ABCC5 and NRF2 were closely related to each other. Expression of ABCC2 and PXR were also closely related, and further related to SLCO1B3 and 1B1 expression.

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Tables:

Table 1: Results for significant correlation test among transporter mRNA expression.

Hierarchical clustering analysis with Pearson correlation as a similarity measurement was performed to identify potential groups of genes with high correlation. * indicates a statistically significant correlation ($p \leq 0.05$) in expression.

	SLCO1B1	SLCO1B3	SLCO2B1	ABCC2	ABCC3	ABCC4	ABCC5	ABCC6	ABCG2
SLCO1B1	1.00								
SLCO1B3	0.53*	1.00							
SLCO2B1	0.23*	0.00	1.00						
ABCC2	0.64*	0.72*	0.42*	1.00					
ABCC3	0.33*	0.51*	0.42*	0.74*	1.00				
ABCC4	-0.24*	-0.49*	0.38*	-0.25*	-0.03	1.00			
ABCC5	-0.10	-0.30*	0.52*	0.05	0.04	0.52*	1.00		
ABCC6	0.55*	0.54*	0.58*	0.70*	0.72*	-0.12	0.11	1.00	
ABCG2	0.19	0.06	0.57*	0.39*	0.45*	0.32*	0.14	0.55*	1.00

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Table 2: Results for significant correlation test among mRNA expression of transcription factors CAR, FXR, NRF2, and PXR.

Hierarchical clustering analysis with Pearson correlation as a similarity measurement was performed to identify potential groups of genes with high correlation. * indicates statistically significant correlation in the expression of corresponding transcription factor, with p value < 0.05.

	CAR	FXR	NRF2	PXR
CAR	1.00			
FXR	0.26*	1.00		
NRF2	0.20	0.12	1.00	
PXR	0.74*	0.37*	0.05	1.00

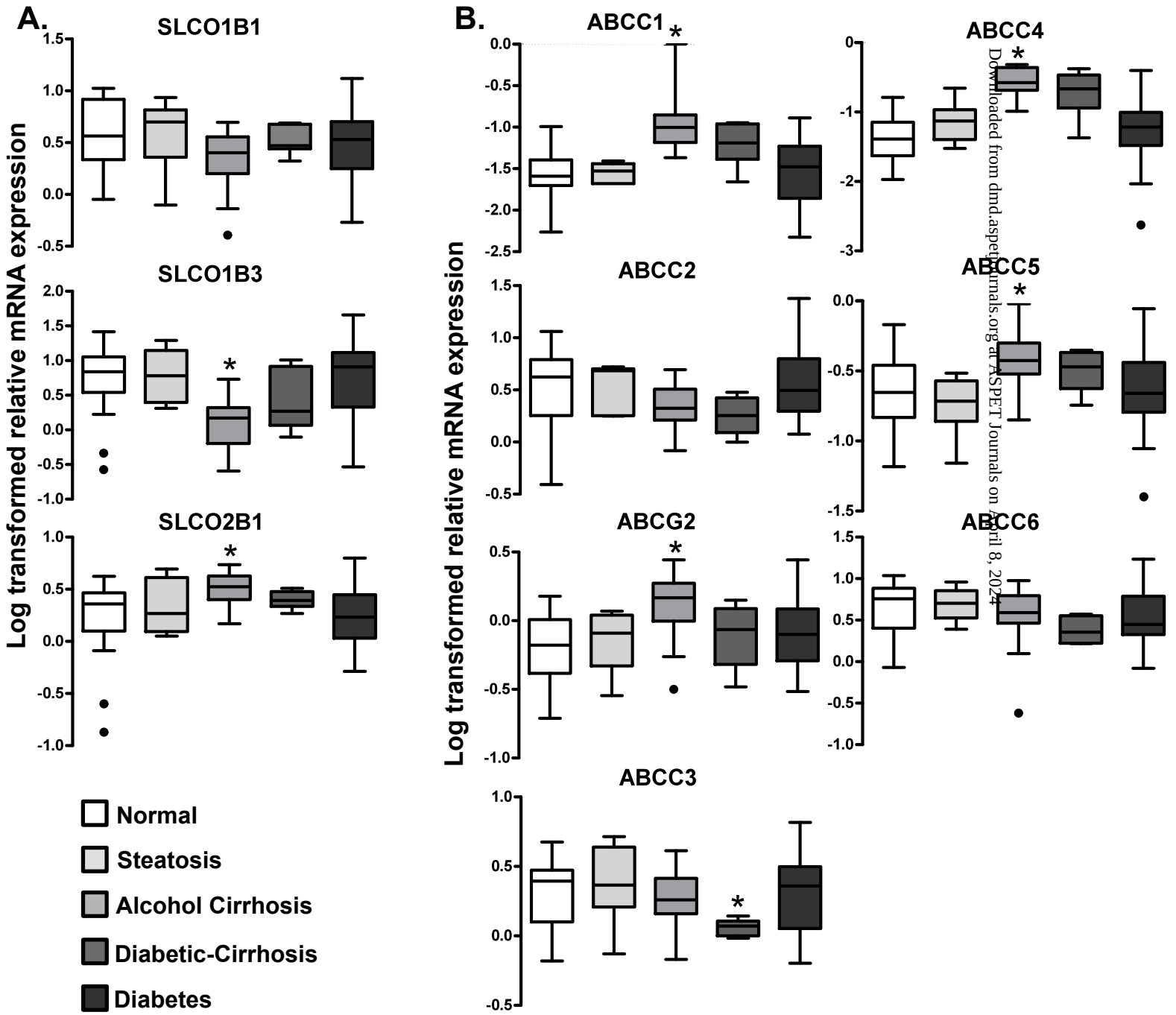

Figure 1

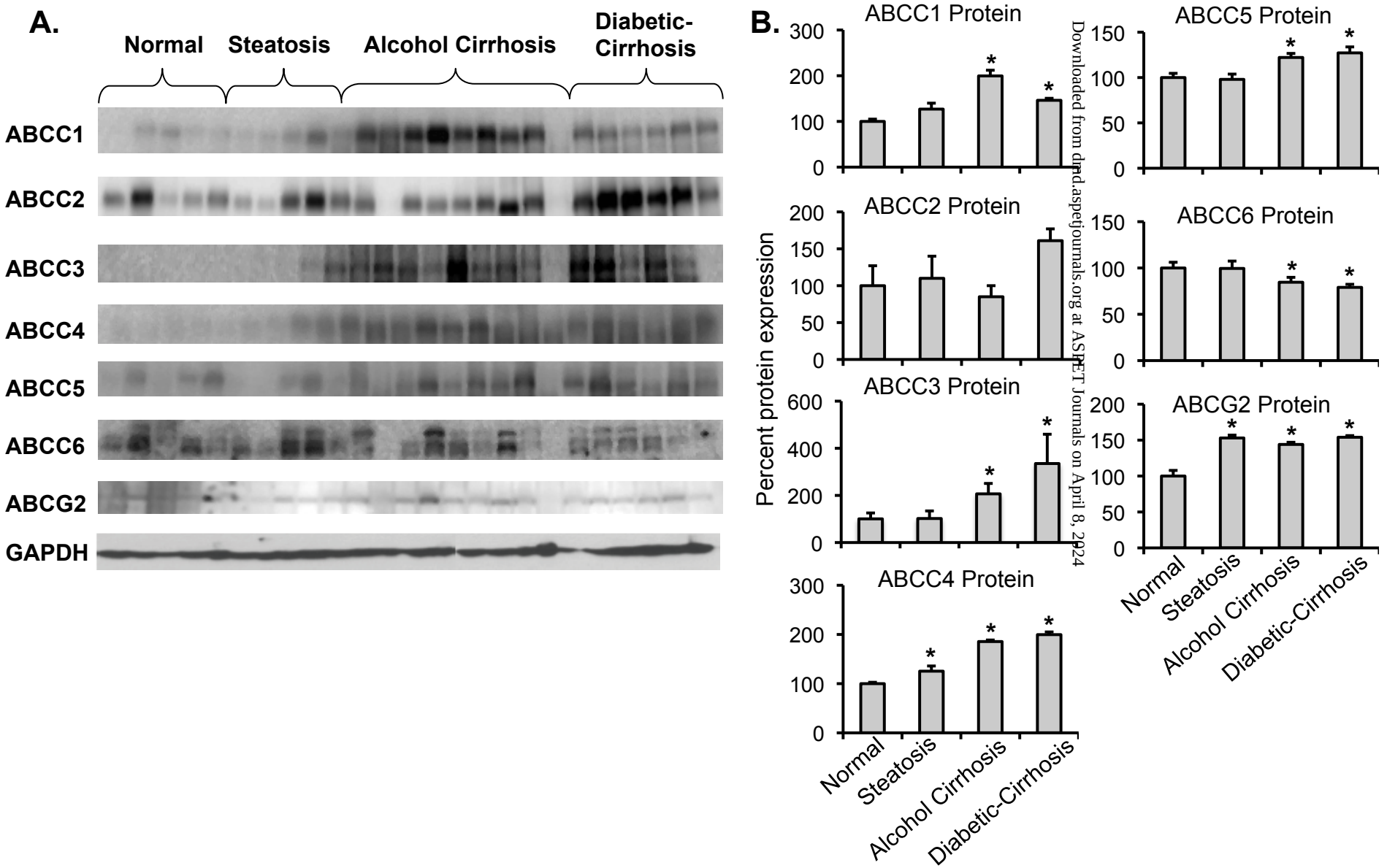

Figure 2

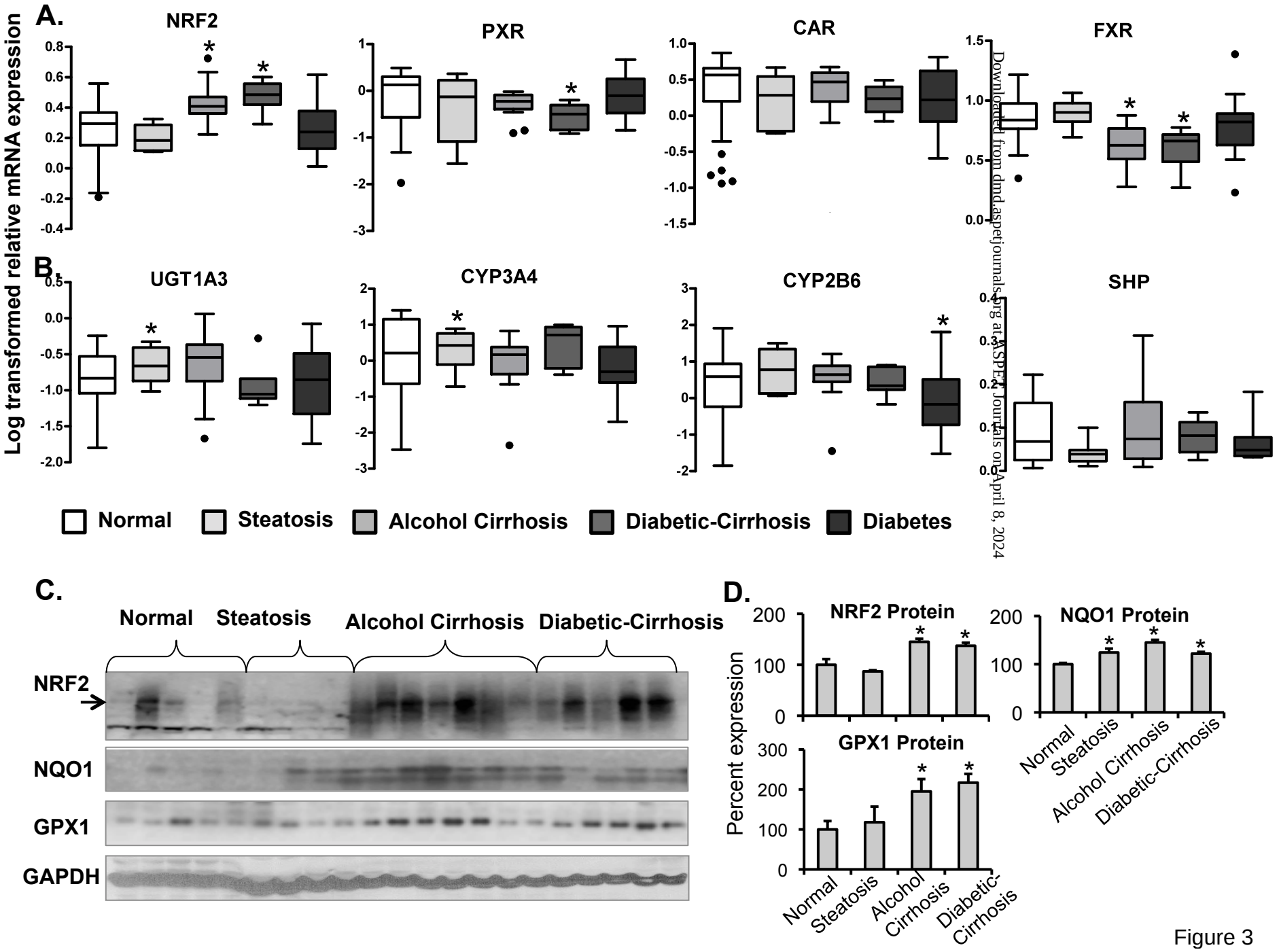

Figure 3

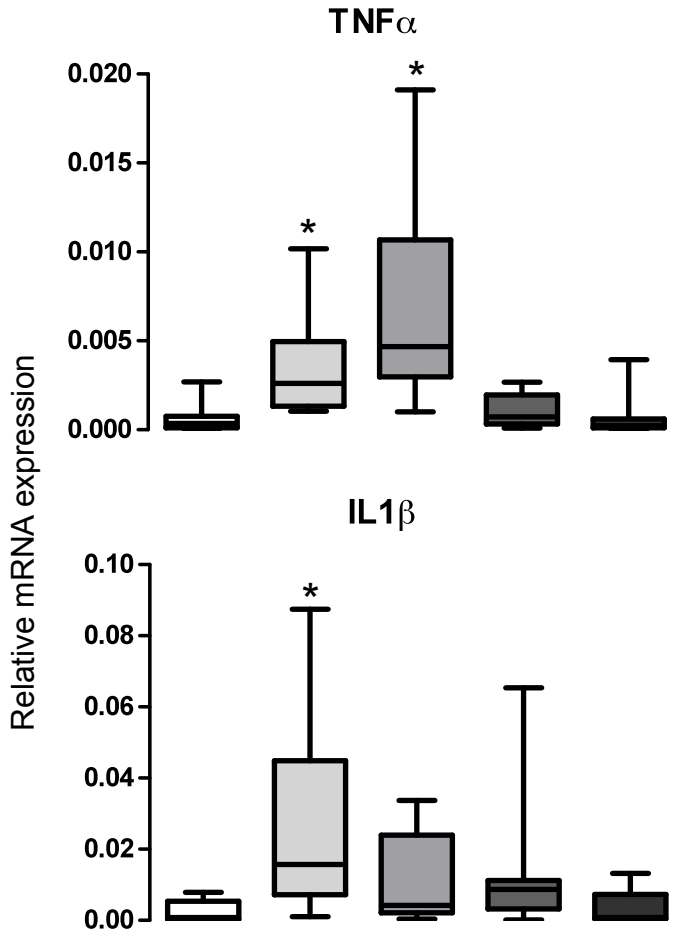

Figure 4

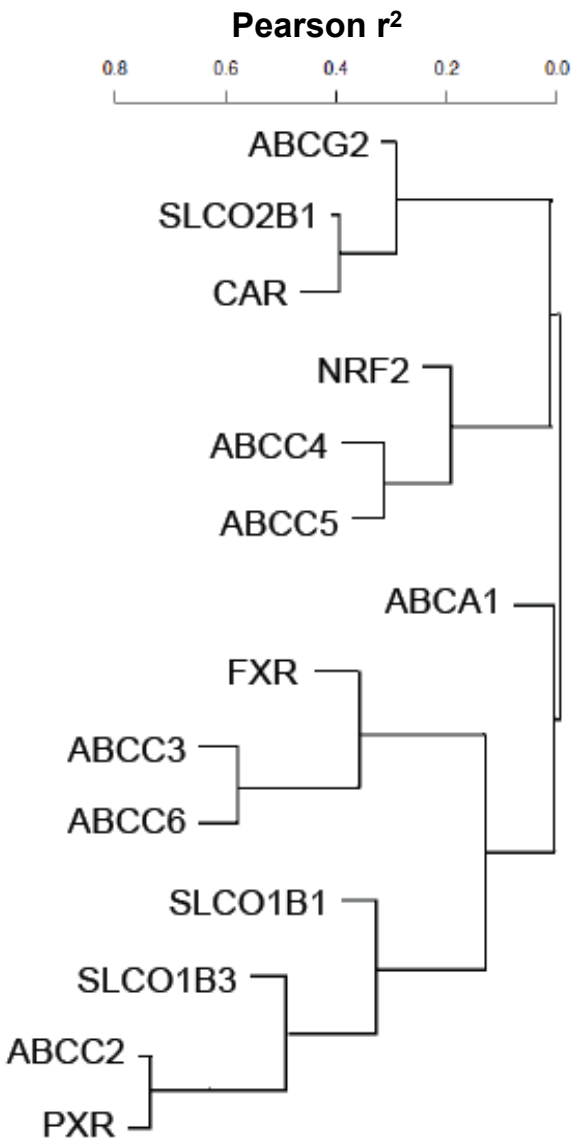

Figure 5

Alcohol Cirrhosis Alters Nuclear Receptor and Drug Transporter Expression in Human Liver

Drug Metabolism and Disposition

Vijay R. More, Qiuqiong Cheng, Ajay C. Donepudi, David B. Buckley, Zhenqiang James Lu, Nathan J. Cherrington, and Angela L. Slitt

Supplementary Table S2: Type, dilution, molecular weight and source information for antibodies used in western blot

Antibody	Type	Dilution	Mol wt	Source
ABCC1	MRPr1	1:2000	~190	Dr. G. Scheffer, VU Medical Center, Amsterdam
ABCC2	M_2III-5	1:600	~190	Chemicon International- Millipore, MA
ABCC3	M_3II-2	1:2000	~180	Dr. G. Scheffer, VU Medical Center, Amsterdam
ABCC4	M_4I-10	1:2000	~160	Dr. G. Scheffer, VU Medical Center, Amsterdam
ABCC5	M_5I-60	1:1000	~160	Dr. G. Scheffer, VU Medical Center, Amsterdam
ABCC6	M_6II-68	1:1000	~165	Dr. G. Scheffer, VU Medical Center, Amsterdam
ABCG2	BXP-53	1:2000	~75	Dr. G. Scheffer, VU Medical Center, Amsterdam
NQO1	Ab2346	1:5000	~30	Abcam, Cambridge, MA
GPX1	AB16798	1:2000	~27	Abcam, Cambridge, MA
NRF2	SC13032	1:1000	~110	Santa Cruz Biotech. Inc, Santa Cruz, CA
GAPDH	D16H11	1:2000	~37	Cell Signaling Technology, Danvers, MA

Article title: Alcohol Cirrhosis Alters Nuclear Receptor and Drug Transporter Expression in Human Liver

Vijay R. More, Qiuqiong Cheng, Ajay C. Donepudi, David B. Buckley, Zhenqiang James Lu, Nathan J. Cherrington, and Angela L. Slitt Drug Metabolism and Disposition

Supplementary Table S1: Gender, ethnicity, age, serum aspartate transaminase, alkaline phosphatase and bilirubin information of the human liver samples used in the study

Sr. No.	Condition	Gender	Ethnicity	Age	Serum Aspartate Transaminase (IU/L)	Serum Alkaline Phosphatase (IU/L)	Serum Total Bilirubin (mg/dL)
1	Normal	F	African American	61	not available	not available	not available
2	Normal	F	Caucasian	57	not available	not available	not available
3	Normal	F	Caucasian	76	not available	not available	not available
4	Normal	F	Caucasian	39	not available	not available	not available
5	Normal	F	Caucasian	55	not available	not available	not available
6	Normal	F	Caucasian	49	not available	not available	not available
7	Normal	M	African American	48	not available	not available	not available
8	Normal	M	Caucasian	21	not available	not available	not available
9	Normal	M	Caucasian	35	not available	not available	not available
10	Normal	M	Caucasian	59	not available	not available	not available
11	Normal	M	Caucasian	44	not available	not available	not available
12	Normal	M	Caucasian	69	not available	not available	not available
13	Normal	M	Caucasian	55	not available	not available	not available
14	Normal	M	Caucasian	73	not available	not available	not available
15	Normal	M	not available	49	not available	not available	not available
16	Normal	M	not available	45	not available	not available	not available
17	Normal	F	Caucasian	60	not available	not available	not available
18	Normal	F	Caucasian	51	not available	not available	not available
19	Normal	M	Caucasian	56	not available	not available	not available
20	Normal	M	Caucasian	69	not available	not available	not available
21	Normal	M	Caucasian	22	not available	not available	not available
22	Normal	M	Caucasian	62	not available	not available	not available
23	Steatosis	F	Caucasian	38	23	not available	not available
24	Steatosis	F	not available	41	0	not available	not available
25	Steatosis	M	Caucasian	40	34	not available	not available
26	Steatosis	M	Caucasian	46	13	not available	not available
27	Steatosis	M	Caucasian	37	75	not available	not available
28	Steatosis	M	Caucasian	31	93	not available	not available
29	Steatosis	M	Caucasian	46	19	not available	not available
30	Steatosis	M	Caucasian	46	186	not available	not available
31	Alcohol Cirrhosis	F	Caucasian	47	50	140	2.8
32	Alcohol Cirrhosis	F	Caucasian	47	553	285	34.5
33	Alcohol Cirrhosis	F	Caucasian	44	240	145	31.3
34	Alcohol Cirrhosis	F	Caucasian	36	41	not available	2.2
35	Alcohol Cirrhosis	F	not available	48	36	116	4.7
36	Alcohol Cirrhosis	F	not available	51	27	97	0.7
37	Alcohol Cirrhosis	F	not available	33	681	56	12.4
38	Alcohol Cirrhosis	F	not available	52	43	165	6.3
39	Alcohol Cirrhosis	M	Caucasian	54	48	147	2.2
40	Alcohol Cirrhosis	M	Caucasian	52	1425	114	4.9
41	Alcohol Cirrhosis	M	Caucasian	50	200	259	not available
42	Alcohol Cirrhosis	M	Caucasian	44	115	122	6.4
43	Alcohol Cirrhosis	M	Caucasian	48	37	174	4.1
44	Alcohol Cirrhosis	M	Caucasian	63	102	258	15.4
45	Alcohol Cirrhosis	M	Caucasian	56	77	121	5
46	Alcohol Cirrhosis	M	Caucasian	55	29	82	2.3
47	Alcohol Cirrhosis	M	not available	42	28	305	1.2
48	Alcohol Cirrhosis	M	not available	33	501	51	18.9
49	Alcohol Cirrhosis	M	not available	46	47	159	2.7
50	Diabetic-Cirrhosis	F	not available	not available	34	180	not available
51	Diabetic-Cirrhosis	M	Caucasian	62	71	79	32.4
52	Diabetic-Cirrhosis	M	Caucasian	39	1720	178	not available
53	Diabetic-Cirrhosis	M	Caucasian	52	30	173	1.1
54	Diabetic-Cirrhosis	M	Caucasian	36	144	222	39.9
55	Diabetic-Cirrhosis	M	Caucasian	65	68	118	not available
56	Diabetic-Cirrhosis	M	Caucasian	50	200	259	13.9
57	Diabetic-Cirrhosis	M	Caucasian	57	215	353	33.7
58	Diabetic-Cirrhosis	M	not available	56	3930	226	6.8
59	Diabetes	F	Caucasian	68	66	199	not available
60	Diabetes	F	Asian	42	405	115	not available
61	Diabetes	F	African American	49	not available	not available	not available
62	Diabetes	F	Caucasian	51	not available	not available	not available
63	Diabetes	F	Caucasian	39	not available	not available	not available
64	Diabetes	F	Caucasian	72	not available	not available	not available
65	Diabetes	F	Caucasian	46	not available	not available	not available
66	Diabetes	F	Caucasian	74	not available	not available	not available
67	Diabetes	F	Caucasian	53	not available	not available	not available
68	Diabetes	F	Caucasian	38	not available	not available	not available
69	Diabetes	M	Caucasian	57	35	124	not available
70	Diabetes	M	Caucasian	48	196	632	not available
71	Diabetes	M	African American	44	not available	not available	not available
72	Diabetes	M	Caucasian	66	not available	not available	not available
73	Diabetes	M	Caucasian	55	not available	not available	not available
74	Diabetes	M	Caucasian	74	not available	not available	not available
75	Diabetes	M	Caucasian	39	not available	not available	not available
76	Diabetes	M	Caucasian	70	not available	not available	not available
77	Diabetes	M	Caucasian	46	not available	not available	not available
78	Diabetes	M	Caucasian	33	not available	not available	not available