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Nonalcoholic fatty liver disease and diabetes is associated with decreased CYP3A4 protein expression and activity in human liver

Rohitash Jamwal[1], **Suzanne M. de la Monte**[2], **Ken Ogasawara**[1], **Sravani Adusumalli**[1], **Benjamin B Barlock**[1], **Fatemeh Akhlaghi**[1]

[1]Biomedical and Pharmaceutical Sciences, College of Pharmacy, University of Rhode Island, Kingston, RI, 02881, USA

[2]Departments of Medicine, Pathology, Neurology, and Neurosurgery, Rhode Island Hospital and the Warren Alpert Medical School of Brown University, Providence, RI 02903, USA

Abstract

Nonalcoholic fatty liver disease (NAFLD) is a major cause of chronic liver disease in the Western population. We investigated the association of nonalcoholic fatty liver disease (NAFLD) and diabetes mellitus on CYP3A4 activity in human liver tissue from brain dead donors (N=74). Histopathologically graded livers were grouped into normal (n=24), nonalcoholic fatty liver (NAFL, n=26) and nonalcoholic steatohepatitis (NASH, n=24) categories. The rate of conversion of midazolam to its 1-hydroxy metabolite was used to assess *in vitro* CYP3A4 activity in human liver microsomes (HLM). A proteomics approach was utilized to quantify the protein expression of CYP3A4 and related enzymes. Moreover, a physiologically based pharmacokinetic (PBPK) model was developed to allow prediction of midazolam concentration in NAFL and NASH patients. CYP3A4 activity in NAFL and NASH was 1.9 and 3.1-fold ($p<0.05$) lower than normal donors, respectively. Intrinsic clearance (CLint) was 2.7 ($p<0.05$) and 4.1 ($p<0.01$) fold lower in donors with NAFL and NASH, respectively. CYP3A4 protein expression was significantly lower in NAFL and NASH donors ($p<0.05$) and accounted for midazolam hydroxylation variability in a multiple linear regression analysis ($\beta=0.869$, $r^2=0.762$, $P<0.01$). Diabetes was also associated with decreased CYP3A4 activity and protein. Both midazolam CLint and CYP3A4 protein abundance decreased significantly with increase in hepatic fat accumulation. Age and gender did not exhibit any significant association with the observed alterations. Predicted midazolam exposure was 1.7 and 2.3-fold higher for NAFL and NASH, respectively, which may result in a longer period of sedation in these patients. Data suggests that NAFLD and diabetes are associated with the decreased hepatic CYP3A4 activity. Thus, further evaluation of clinical consequences of these findings on the efficacy and safety of CYP3A4 substrates is warranted.

Corresponding author at: Fatemeh Akhlaghi, Ph.D., Clinical Pharmacokinetics Research Laboratory, Department of Biomedical and Pharmaceutical Sciences, The University of Rhode Island, 495A College of Pharmacy, 7 Greenhouse Road, Kingston, RI 02881, United States. fatemeh@uri.edu.

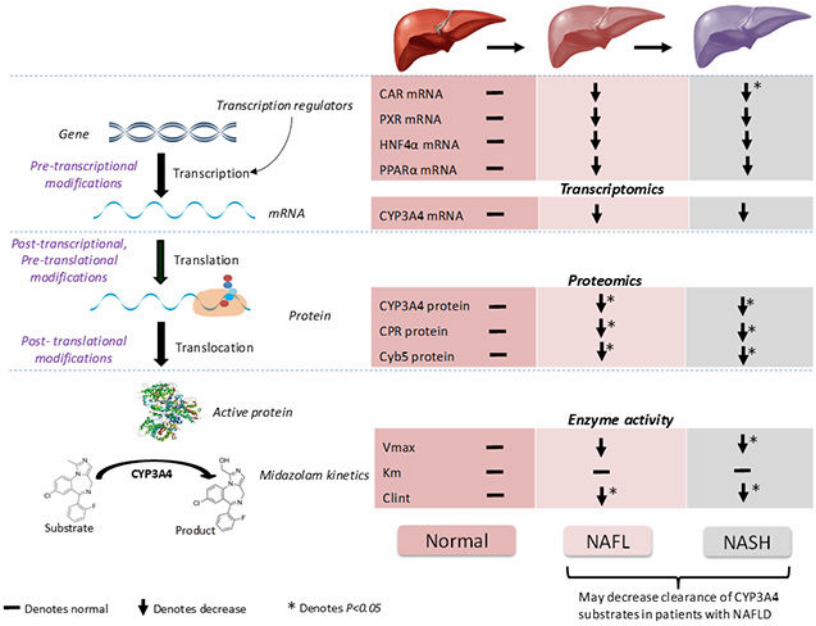
Conflict of interest

None of the authors have any conflict of interest to declare.

Supporting information

The supporting tables and figures for the manuscript are listed below.

Graphical Abstract



Keywords

CYP3A4; diabetes; drug metabolism; midazolam; nonalcoholic fatty liver disease; nonalcoholic steatohepatitis; PBPK; pharmacokinetics; proteomics

INTRODUCTION

The prevalence of nonalcoholic fatty liver disease (NAFLD) is rising at an alarming rate in populations with diabetes and obesity. The clinical diagnosis of NAFLD is based on limited or no consumption of alcohol, evidence of hepatic steatosis based on either biopsy or imaging, and the exclusion of other causes of liver disease. NAFLD is sub-categorized histopathologically as non-alcoholic fatty liver (NAFL), which is characterized by lipid accumulation in hepatocytes, or non-alcoholic steatohepatitis (NASH), which is associated with hepatic steatosis with inflammation and ongoing inflammatory or degenerative injury to hepatocytes [1, 2]. Significant lobular inflammation, hepatic fibrosis, and hepatocyte necrosis present in NASH can progress to life-threatening liver cirrhosis and hepatocellular carcinoma (HCC).[3]

Over 64 million people in the United States are projected to have NAFLD, with an estimated economic burden of about $103 billion, and these costs are highest among patients aged 45-65 year.[4] Presence of NAFLD is highly correlated with insulin resistance and diabetes.[5] Diabetes and NAFLD share common underlying pathophysiological processes including insulin resistance, hyperglycemia, dyslipidemia, inflammation and other cardiovascular conditions.[6] A retrospective analysis of the hospital admission of patients with type 2 diabetes showed that the relative risk of NAFLD among these patients was 3-times higher in men and 5-times higher in women than those without diabetes.[7]

Despite the widespread prevalence of NAFLD, no specific pharmacological therapies are available for its treatment; however, management of associated conditions including obesity, diabetes, and hyperlipidemia are often achieved pharmacologically or through lifestyle intervention.[8–10] CYP3A enzymes metabolize most of the drugs prescribed for these comorbidities.[11] Moreover, CYP3A enzymes are also responsible for the metabolism of 30-50% of all drugs available in the market.[11, 12] CYP3A4 and CYP3A5 proteins of this superfamily of enzymes are expressed in human liver as well as extrahepatic tissues including intestine.[13] Moreover, CYP3A4 plays a significant role in the catabolism of carcinogens (aflatoxin B1), and various endogenous steroids (progesterone, testosterone, cortisol and bile acids).[14–17] Additionally, the enzyme is involved in the biotransformation of cholesterol, and the plasma concentration of cholesterol 4β-hydroxy has been used as an endogenous marker of CYP3A4 activity.[18] CAR, PXR, HNFα, and PPARα are some of the transcription factors which has been reported to modulate the expression of CYP3A4.[19, 20]

A perturbation in CYP3A4 activity associated with diabetes, NAFLD or both is likely to alter the clearance of some drugs thereby changing the efficacy or safety of CYP3A substrates. Current studies in rodent models of the disease state are conflicting as it has been widely known that most models fail to recapitulate the complex pathophysiology of human NAFLD fully. [21–23] In rat models, reduced or elevated expression and activity of Cyp3a in steatosis and NASH has been reported [24–28]. High-fat and sucrose diets in Wistar rats resulted in an initial decrease in Cyp3a protein level after two weeks, but protein expression was increased after eight weeks [29]. Similarly, studies in mice models are inconsistent, and the outcome is related to diet and the type of animal model. [30–32]

Genome-Wide Association Studies (GWAS) in human NAFLD patients found no significant changes in drug metabolism genes between normal and steatotic livers at mRNA expression level.[33, 34] However, these results do not capture the post-transcriptional and post-translational modifications which may alter protein expression or activity. The results of studies in human liver examining the impact of NAFLD on CYP3A4 activity are heterogeneous and lack agreement.[35–38] Fisher et al. found a trend of decreasing CYP3A4 protein expression with the severity of NAFLD, but no change in enzyme activity was observed. [35] However this study was performed in a small sample size of livers (steatosis =10, NASH=10) and no information on the presence of diabetes or ethnicity of the donor was provided. In contrast, Wooley et al. found significantly lower CYP3A4 activity in human NASH subjects but again the presence of diabetes was unknown in this study. [38] Our group previously reported a significant reduction of CYP3A4 activity in human livers. [39] However, the sample size of our earlier investigation was small, and the presence of NAFL or NASH was unknown. [39]

Therefore, using a large repository of human liver tissue (N=74), well-characterized with respect to the presence of diabetes and NAFLD, we aimed to verify which disease condition influence the expression and activity of CYP3A4. CYP3A4 functional activity and intrinsic clearance of midazolam was evaluated in HLM. The goal of the studies using microsomes was to determine the intrinsic clearance to enable building an in vitro in vivo extrapolation (IVIVE) model. As protein expression is an important factor for IVIVE, we determined the expression of CYP3A4 and related proteins in HLM using mass spectrometry. Furthermore,

we developed a physiologically based pharmacokinetic (PBPK) model and predicted midazolam exposure in virtual populations of NAFL and NASH patients.

EXPERIMENTAL SECTION

Materials and reagents.

Midazolam (MDZ), 1-hydroxy midazolam (1′-OH MDZ), and 1-hydroxy midazolam-D_4 (1′-OH MDZ-D_4) were purchased from Cerilliant Corporation (Round Rock, TX). MS-grade formic acid, acetonitrile, and methanol were obtained from ThermoFisher Scientific (Waltham, MA). NADPH tetrasodium salt was from Calbiochem (EMD Millipore, Billerica, MA). OxiSelect TBARS assay kit (Malondialdehyde quantification) was purchased from Cell Biolabs, Inc., San Diego, CA and Amplex® Red Cholesterol Assay Kit was from ThermoFisher Scientific, Waltham MA. All other reagents and solvents used in the study were of analytical grade.

Human liver bank.

A novel human liver tissue repository (N=106) was created in our laboratory from hepatic tissue purchased from Sekisui XenoTech LLC (Kansas City, KS). The identity of donors was not known thereby the study was designated as Institutional Review Board (IRB) exempt category 4. Age, gender, ethnicity, the cause of death, cold ischemia time, liver and body weight was available for ≥ 95% of the samples. While being accessible, smoking and alcohol consumption was not assessed for effect on CYP3A4 activity due to reasons discussed later in the text. Detailed donor demographics are given in Table 1. The primary objective of the work was to study the effect of NAFLD and diabetes on CYP3A4 activity and protein levels. Therefore, only samples that were homozygous for *CYP3A5*3/*3* (n=74) were included in this study. The results and findings of this study are thus limited to Caucasian male and female population. Moreover, five donors were identified with *CYP3A4*22* variant and one possessed *CYP3A4*1B* (Table 1).

Histological grading and study grouping.

Liver tissue was graded for steatosis, lobular inflammation, hepatocyte ballooning, and fibrosis by a physician specializing in histopathology (Suzanne Delamonte, MD). The standardized scoring protocol assessed the presence and severity of hepatocellular steatosis, lobular inflammation, ballooning degeneration, and fibrosis.[40] Formalin-fixed paraffin-embedded histological sections (5 μm thick) of the liver, stained with hematoxylin and eosin dyes, were used to grade the severity of the disease. In brief, the slides were coded and scored concerning the abundance and distribution of hepatic steatosis, lobular inflammation, hepatocellular ballooning degeneration, and fibrosis. Steatosis was graded as 0 (<5%), 1 (5-33%), 2 (34-66%), or 3 (>66%), reflecting the cross-sectional areas of the section showing hepatocytes with steatosis. Lobular inflammation was graded as 0 (absent), 1 (<2 foci/200x microscopic field), 2 (between 2 and 4 foci/200x microscopic field), or 3 (>4 foci/200x microscopic field). Hepatocyte ballooning degeneration was graded as 0 (absent), 1 (rare, scattered cells) or 2 (readily detected). Fibrosis grading was simplified relative to the original report and graded as 0 (absent), 1 (mild and delicate in the perisinusoidal regions), 2 (conspicuously present in perisinusoidal and periportal regions), 3 (bridging fibrosis), or 4

(cirrhosis, which requires bridging fibrosis and regenerative nodules). The final scores represent the summed sub-scores. Steatosis was confirmed by Oil Red O staining of cryostat sections (10 microns) of the same liver samples, and fibrosis was confirmed by Sirius red staining of adjacent formalin fixed paraffin-embedded tissue sections.[41] A composite of different histological sections of representative liver samples from different groups is given in Fig. 1.

Subsequently, livers were categorized as normal, NAFL, and NASH based on the scoring algorithm described in supplemental figure I.[42] Detailed histological characteristics of donors are given in supplemental table I.

Malondialdehyde and cholesterol estimation.

Quantification of malondialdehyde (MDA) in donor liver homogenate was determined using OxiSelect TBARS assay kit according to manufacturer instructions (Cell Biolabs, Inc., San Diego, CA). Total liver cholesterol was estimated using Amplex® Red cholesterol assay kit according to manufacturer's instruction (ThermoFisher Scientific, Waltham, MA).

Quantification of mRNA.

Total RNA from liver tissues was isolated using the RNeasy mini kit (Qiagen Inc., Valencia, CA). Subsequently, the total cellular RNA was reverse-transcribed, and the cDNA was used for real-time PCR analysis. The mRNA expression of hepatic CYP3A4 and the relevant transcription factors were quantified by real-time PCR using SYBR Green Master Mix on 7500 Real-Time PCR system (Applied Biosystems, ThermoFisher Scientific, MA). Human 18S ribosomal RNA (rRNA) was used as an internal control for relative expression of data. The information on PCR primers used is available in supplemental table II.

CYP3A4 activity assay.

Microsomes were prepared from liver samples as described previously in detail.[43] CYP3A4 activity was assessed by formation of 1-hydroxy midazolam in HLM using midazolam as probe substrate.[44] Enzymatic incubations were carried out in 100 mM potassium phosphate buffer containing 3 mM $MgCl_2$ (pH 7.4). Microsomal protein concentration used was 50 μg/mL and concentrations of midazolam were 0, 0.2, 1, 2.5, 5, 10, 25 μM. The addition of NADPH initiated the reaction, and the incubations were carried out in a shaking water bath (75 rpm) kept at 37°C. After 20 min, the reaction was terminated by addition of ice-cold acetonitrile containing 0.5% formic acid and internal standard (1-hydroxymidazolam-D_4, 50 ng/mL). Subsequently, samples were centrifuged at 2000 *g* for 5 min at 4°C. The supernatant was collected, and 5 μL was injected for quantification of 1-hydroxy midazolam using the UPLC-MS method described below. CYP3A4 activity (Vmax; maximum rate of reaction) was expressed as pmol/mg microsomal protein.

Quantification of 1-hydroxy midazolam.

Samples were analyzed using a previously published method with some modifications to chromatography method as described below [44]. Chromatographic separation was performed using a gradient elution mode using 10 mM ammonium acetate and 10% methanol (A) and acetonitrile (B) at the flow rate of 400 μL/min. The linear gradient started with 25% B until

0.5 min, 60% B at 1.5 min, 90% B at 3 min before returning to 25% B at 4 min. All other mass spectrometer parameters were same as previously described.[44]

Quantification of proteins using mass spectrometry.

Protein levels of CYP3A4, NADPH-cytochrome P450 reductase (CPR), and Cytochrome b5 (Cyb5) in human liver microsomes were determined using mass spectrometry and "Total Protein Approach".[45] Microsomal fractions were digested with trypsin and analyzed in Data-Dependent Acquisition (DDA) mode on SCIEX TripleTOF 5600+ mass spectrometer (SCIEX, Concord, CA). The raw data files from one of our previous study were analyzed using MaxQuant (ver 1.5.2.10).[43] The specifics of protein digestion and mass spectrometry analysis were previously described in the literature.[43, 45] The proteins were searched on Andromeda search engine against UniProt human protein database (updated Oct 2016) at 1% false discovery rate (FDR).[46] Cysteine carbamidomethylation was selected as fixed modifications for the search. Label-free quantification (LFQ) was performed with a ratio count of 1 and maximum of two missed cleavages were allowed. All the other MaxQuant settings were kept as default values. The absolute protein levels were calculated using "Total Protein Approach" from LFQ intensities obtained from MaxQuant using the equations given below[45].

$$Total\ protein\ (p) = \frac{MS\ signal\ (p)}{Total\ MS\ signal}$$

$$Protein\ concentration\ (p) = \frac{MS\ signal\ (p)}{Total\ MS\ signal\ x\ MW\ (p)} [\text{mol/gram total protein}]$$

where MS signal (p) refers to total LFQ signal intensity for CYP3A4, CPR or Cyb5. Total MS signal refers to the total LFQ intensity of all the proteins and MW represents the molecular weight of respective protein.

Modeling of enzyme kinetics data.

In vitro CYP3A4 kinetics data were fitted using Prism® version 6 (GraphPad Software Inc., La Jolla, CA) into a nonlinear least-squares regression equation given below [47].

$$v = \frac{V_{max} \times S}{K_m + S \times \left(1 + \frac{S}{K_S}\right)}$$

Where S represents the concentration of substrate, v is the velocity of 1-OH midazolam formation; K_m is Michaelis-Menten constant (substrate concentration required for an enzyme to reach one-half its maximum velocity), and K_s is inhibition constant. V_{max} (maximum rate of product formation) and K_m were estimated from the equation, and apparent *in vitro* intrinsic clearance (CLint, app) was calculated as V_{max}/K_m. Intrinsic clearance for whole liver (CLint, whole liver) was calculated using the equations given

below and was expressed as L/min. MPPGL denotes the yield of membrane proteins per gram of liver.

$$CLint(wholeliver) = CLint, app \times MPPGL \times Liverweight$$

SimCYP based PBPK simulation.

A physiologically based pharmacokinetic (PBPK) model was developed in SimCYP population-based simulator (ver 15, Certara LP, Sheffield, UK) using CYP3A4 protein and midazolam enzyme kinetic parameters. A virtual Caucasian population (Sim-NEurcaucasian) with an equal proportion of males and females, 20-65 year old, was selected for simulations. A minimal PBPK model was utilized for estimating plasma concentration-time profiles. Portal and arterial blood flow were the same for all the populations given a lack of data on hepatic blood flows in NAFL and NASH. An intravenous bolus dose of 5 mg midazolam was given, and default SimCYP compound file was used. In vitro Vmax and Km values were substituted for a respective study group in the “whole organ metabolic clearance” tab implemented in SimCYP. Default CYP3A4 phenotype values for the Caucasian population was replaced with protein concentrations determined in this study. Five virtual trials with 50 subjects per study were used for prediction of systemic midazolam concentration. All other parameters were kept as the default values.

Statistical analysis.

Statistical analysis was performed with SPSS version 24 (IBM Analytics, Armonk, NY), and Prism® version 6 (GraphPad Software Inc., La Jolla, CA) was used for graphs, V_{max} and K_m calculations. Descriptive statistical values in tables are reported as mean ± standard error (SE) unless otherwise stated. Mann-Whitney U test (2-tailed) was used to compare the effect of gender. Non-parametric Kruskal-Wallis test (2-tailed) without multiple corrections was used when studying three or more groups. The correlation was analyzed using nonparametric Spearman correlation analysis. Linear and multiple regression analysis were used to determine the contribution of predictors toward explaining variability in CYP3A4 activity. $P < 0.05$ was considered significant for all the statistical tests and correlation analysis.

RESULTS

CYP3A4 activity and protein expression are decreased in NAFLD.

Significantly lower CYP3A4 activity (Vmax expressed as pmol/min/mg protein) was observed in microsomes from NAFLD donors (Fig. 2). HLM from NASH donors exhibited 3.1-fold lower midazolam Vmax as compared with normal donors (Table 2). Midazolam Vmax was 1.9-fold lower in HLM from NAFL donors, but the effect was not statistically significant (P>0.05). CLint (L/min) was significantly lower intrinsic clearance in NAFL (2.7±0.9, P<0.05) and NASH (1.8±0.6, P<0.01) as compared to normal (7.3±1.8, Fig. 3). Michaelis-Menten constant (K_m) was comparable in different study groups and ranged from 1.6 to 2.3 μM (Table 2).

Mean protein levels of CYP3A4 decreased with progression of disease (*P<0.05*, Fig. 3). CPR and Cyb5 protein levels were significantly lower (*P<0.05*) in HLM from NAFLD donors (Table 3). The progression of disease from NAFL to NASH reduced the CYP3A4 mRNA expression (Fig. 4). However, the decrease was not significant. The levels of transcription factors, PXR, CAR, HNF4α and PPARα mRNA, decreased with disease progression (Fig. 4). The reduction in CAR mRNA level was significantly different between normal and NASH donors (*P<0.05*).

CYP3A4 activity and protein expression are decreased in diabetes and NAFLD.

Given the high prevalence of NAFLD in patients with diabetes, we further studied the combined effect of the insulin resistance and fatty liver on CYP3A4 protein and activity. The levels of CYP3A4 activity, protein and mRNA expression and relevant transcription factors are summarized in Table 4. We observed that the effect of NAFLD was more prominent and statistically significant in HLM from diabetic donors. For non-diabetic donors, the study parameters showed a trend of reduction in disease state but were not statistically significant except CPR. In contrast, CYP3A4 activity and Clint was significantly lower in HLM from diabetic NAFL and NASH donors. Similarly, a significant decrease in CYP3A4, CPR, and Cyb5 was also observed in diabetic donors with NAFLD. Interestingly, while mRNA expression decreased in both disease states, the mean differences failed to achieve statistical significance.

CYP3A4 activity and protein expression decrease with increase in liver fat.

The impact of varying grades of steatosis on CYP3A4 activity, protein and mRNA levels was also evaluated. When the donors were categorized based on the severity of steatosis, a significant reduction in CLint was observed (Fig. 5). CYP3A4 activity decreased with increase in liver fat content, but the decline was not significant due to extensive variability. CYP3A4, CPR, and Cyb5 protein levels also reduced with an increase in the severity of steatosis (Fig. 5, supplemental figure III).

Effect of age.

The average age of normal donors was similar in NAFL and NASH donors (Table 1). Donor age showed no significant correlation with CYP3A4 activity ($r=-0.143$, $P>0.1$), CYP3A4 protein ($r=-0.101$, $P>0.1$), and CLint ($r=-0.228$, $P>0.1$, Supplemental figure IV**)**. The association with age was also insignificant for CYP3A4 mRNA.

Effect of gender.

Almost equal number of male and female donors were included in this study (males=36, females=38, Supplemental table III). In general, males exhibited marginally higher CYP3A4 activity, CLint, protein and mRNA levels than female donors. However, no significant association (*P>0.1*) was observed between gender and CYP3A4 activity, Clint as well as CYP3A4, CPR and Cyb5 protein abundance. The relationship between gender and CYP3A4 and related proteins was also examined, and no significant correlations were found (Supplemental table III).

Correlation between activity, mRNA and protein levels.

The rate of 1-hydroxymidazolam formation showed a significantly positive correlation with CYP3A4 protein and mRNA levels. Similarly, CYP3A4 protein and mRNA expression levels exhibited significant positive correlation **(**supplemental figure V**)**. A significant but moderate correlation was found between CPR protein and CYP3A4 activity (r=0.446, P <0.01), and CYP3A4 protein (r=0.547, P <0.01). The correlation between Cyb5 protein and CYP3A4 activity (r=0.463, P <0.01), and CYP3A4 protein (r=0.592, P <0.01) was moderate and significant. The association between two CPR and Cyb5 was significant as well (r=0.607, P <0.01). Correlation plots can be found in supplemental figure V.

Linear regression analysis of protein and activity.

Univariate linear recreation analysis was used to determine how much of the variability in CYP3A4 activity was accounted by CYP3A4, CPR, and Cyb5 protein levels. We found that CYP3A4 (r^2=0.761, *P<0.01*), CPR (r^2=0.331, *P<0.01*) and Cyb5 (r^2=0.197, *P<0.01*) protein levels were significant predictors of the CYP3A4 activity in HLM. However, multiple linear regression analysis returned a regression coefficient (r^2=0.762, *P<0.01*) which was marginally better than the coefficient returned by univariate linear regression between CYP3A4 protein and activity (r^2=0.761). Standardized beta coefficients of multiple regression models for CYP3A4 protein (β=0.869, P<0.01) explained most of the variability in midazolam hydroxylation activity, whereas CPR protein (β =−0.062, *P>0.1*) and Cyb5 protein (β =−0.069, *P>0.1*) revealed the minimal contribution of each predictor to the model.

SimCYP based PBPK model.

The simulated plasma profile of midazolam was in good agreement with the observed profiles previously reported in the literature so as the values of area under the concentration-time curve (AUC0-24), maximum midazolam concentration (Cmax) and clearance (CL).[48] A 1.8 and 2.3-fold increase in exposure (based on AUC) was found for NAFL and NASH populations, respectively (supplemental table IV). Predicted plasma concentration of midazolam in a Caucasian population with CYP3A4 phenotype and enzyme kinetic parameters is shown in Fig. 6. The predicted intravenous clearance (CL) of midazolam in normal, NAFL and NASH group was 16.6, 9.4 and 8.6 L/h, respectively (supplemental table IV). The pharmacokinetic parameters when accounting for insulin resistance (diabetes) and fatty liver (NAFLD) are given in supplemental table V.

DISCUSSION

Our studies suggest downregulation of CYP3A4 protein and activity in NAFLD. While some findings were not statistically significant for NAFL, a trend of reduction was observed. This pattern achieved significance for HLM from NASH donors suggesting that the decrease in CYP3A4 activity and CYP3A4 protein continues with the severity of disease as it progresses from benign stage to NAFL and NASH. We also found that insulin resistance along with steatosis appears to provide a double-blow leading to decreased CYP3A4 protein and activity. Similar substrate affinity (Km) among the groups indicate that the differences seen in the velocity of the reaction (1-hydroxy midazolam formation) were in fact due to an altered enzyme level rather than its affinity for the substrate. Multiple linear regression

modeling showed that the variability in midazolam hydroxylation was accounted mainly by CYP3A4 protein in HLM. SimCYP based PBPK model was in good agreement for the healthy population when *in vitro* parameters from this study were used.

Woolsey and colleagues found that midazolam concentrations in human subjects with NASH were significantly higher as compared to control subjects (indicating reduced CYP3A activity).[38] Fisher and colleagues suggested a decrease in CYP3A4 expression and functional activity with the progression of NAFLD, but this difference was not statistically significant.[35] The same study reported that the mRNA expression was not different between NAFLD groups. We found that the mRNA expression of CYP3A4 and its transcription factors (CAR, PXR, HNF4α, and PPARα) was reduced in livers from NAFLD donors, but the effect was statistically insignificant except for CAR mRNA. In contrast, the level of protein expression was significantly lower in such donors possibly indicating the involvement of transcriptional and translational mechanisms in down-regulation of CYP3A4 activity.

Two primary inducible nuclear transcription regulators of CYP3A4 mRNA expression in human, PXR and CAR, are widely affected by dietary, genetic, environmental and pathological factors.[49] Similar PXR mRNA levels between fatty and normal human liver microsomes were previously reported.[36] HNF4α was identified as a critical constitutive regulator of PXR and CAR-mediated transcriptional induction of CYP3A4.[50] Conversely, we found a decrease in HNF4α levels, but the alteration was statistically insignificant between the three groups. Interestingly, a study on PXR-knockout rat model indicated that down-regulation of hepatic CYP450s via a PXR-independant mechanism.[51] It could partially explain why the PXR mRNA levels were not significantly different in our study despite a discernible decrease in the disease state. Recently, Woolsey et al. reported that CYP3A4 down-regulation might be due induced fibroblast growth factor 21 (FGF21) leading to reduced PXR localization and binding to the CYP3A4 proximal promoter.[52] While some have reported elevated CAR in the pathogenesis of NASH in mice, other studies suggest a downregulation of CAR.[53, 54] We also found that CAR mRNA expressed was decreased with progression of NAFLD. PPARα governs transport and β-oxidation of fatty acid in the liver in addition to regulation of inflammatory response. We found different levels of PPARα in our groups, but the effect was insignificant. Due to lack of data, we speculate that tandem decrease in levels of transcription factors may result in significant downregulation of some target proteins.

Diabetes and insulin resistance are associated with NAFLD with up to 70% patients having been reported to share both these comorbidities.[55, 56] Our lab has reported the effect of diabetes on CYP3A4 expression and activity in human liver, but the impact of NAFLD was not studied.[39] In this study, we found that while there was a decrease in CYP3A4 activity and protein, the effect was statistically insignificant in HLM from non-diabetic donors. In contrast, NAFL and NASH donors with diabetes showed a statistically significant decrease in activity, protein expression, and midazolam clearance. Interestingly, we found that diabetic, normal donors exhibited marginally higher CYP3A4 activity and Clint as compared to non-diabetic normal donors. We speculate that this anomaly could be possibly attributed to pharmacological agents that these diabetic donors might be receiving. Our lab is currently

pursuing a challenging project to find out the exposure to different drugs at the time of death in our liver bank. Based on the data, we speculate that the heightened reduction of activity and midazolam Clint in diabetic subjects may be due to the double-punch which insulin resistance appears to trigger in steatotic livers. It is also supported in part by lack of statistically significant decrease in CYP3A4 activity in non-diabetic livers.

Kolwankar and colleagues found an independent association between hepatic steatosis and reduced CYP3A activity which decreased with the severity of steatosis.[36] Another study in human hepatocytes isolated from macrosteatosis livers found a significant reduction in CYP3A4 activity without any alteration in CPR levels in microsomes from steatotic and nonsteatotic livers.[57] We also found that livers with >5% hepatic fat were associated with the low CYP3A4 activity and intrinsic clearance of midazolam in HLM.

Previous studies have reported a higher amount of protein, mRNA and CYP3A4 activity in female livers.[13] In contrast, we found that males exhibited slightly higher CYP3A4 level than females. A few clinical studies have reported that women have higher CYP3A4 activity than men based on pharmacokinetic studies with cyclosporine, erythromycin, and midazolam.[58, 59] Conversely, studies with cyclosporine and midazolam suggest an insignificant gender difference in metabolism of these drugs. As CYP3A4 and P-glycoprotein 1 (Pgp) share a large number of common substrates; increased metabolism in females may in part be due to lower Pgp activity in canalicular membrane rather than the CYP3A4 activity in the endoplasmic reticulum.[60] Schuetz reported that Pgp in women was almost half the levels of that in men.[61] Moreover, another study in HLM found that median CYP3A4 content was 2-fold higher in women than men with the *CYP3A5*3/*3* livers ($P<0.05$).[62]

Reports on the effect of NAFLD on NADPH-cytochrome P450 reductase (CPR) are lacking. The expression of CPR reduced significantly with progression of disease from NAFL to NASH. Multiple linear regression analysis with CYP3A activity as the dependent variable revealed that CPR protein content does not account for additional variability when CYP3A4 protein is present as a predictor variable. These results are in line with a previous report which found that CYP3A variability was independent of CPR protein level.[63]

Oxidative stress has been found to be a critical factor associated independently with NAFLD.[64, 65] We found that livers from NAFLD donors had higher malondialdehyde levels compared to normal donors suggesting increased oxidative stress (Table 1). Additionally, liver samples from NALFD donors exhibited a significantly higher amount of cholesterol as compared to normal donors (Table 1). Increased liver cholesterol leads to activation of Kupffer and stellate cells in the liver, thereby promoting inflammation and fibrogenesis.[66] Production of reactive oxygen species during NAFLD may lead to lipid peroxidation which stimulates subsequent activation of stellate cells resulting in fibrogenesis [67, 68].

Studies in the last decade have suggested a strong relationship between metabolic diseases like NAFLD and microRNA expression in human liver.[69, 70] These miRs can regulate expression and mRNA stability function, in addition to the regulation of lipid metabolism, inflammation, and apoptosis.[71, 72] Significantly, upregulated miR-155 levels in cirrhotic

livers showed a strong negative correlation with CYP3A activity.[73] Moreover, miR-27b was found to suppress the translation of CYP3A4 protein without affecting the mRNA levels of the enzyme.[74] We speculate elevated miR species to be one of the factors responsible for a contrast of mRNA and protein expression levels in NAFLD.

The information on any other underlying disease or drug use by donors at the time of their death was not available. The data on prior drug usage, alcohol consumption, and smoking can be challenging to interpret, and it is naïve to assume that such information provided by the vendor is accurate.[75] Contrary to this, data on age and gender can be considered reliable. Therefore, we did not evaluate the effect of smoking and alcohol consumption on the CYP3A activity and clearance of midazolam.

The performance of PBPK model in the normal population was used to estimate the accuracy of predictions made in the normal population.[48] Compared to normal population, a 2.3 fold higher midazolam exposure was predicted by the model for NASH population (supplemental table IV), which is consistent with 2.4-fold higher systemic midazolam concentration reported in a clinical study with subjects with NASH.[38] PBPK model also suggested prolonged sedation in NAFLD patients with compromised liver function. An average midazolam effective concentration (EC50) of 68.7 ng/mL (10.9-165.0, 95% CI) for a Ramsay score between 3-5 was recently reported in a population-based pharmacodynamic model in Caucasians.[76] Considering this, PBPK simulation in NAFLD population showed that the midazolam concentration would remain over EC_{50} for extended time compared to the normal population (Fig. 6). It was reported that subjects with alcoholic cirrhosis had a significantly lower elimination half-life of midazolam compared to healthy subjects.[77] Indeed, Li et al. have reported that patients with the severe liver disease were more sensitive to midazolam and achieved loss of consciousness at much lower systemic concentration compared to subjects with normal hepatic function.[78] Therefore, we can speculate that NAFL and NASH patients may also be more sensitive to midazolam that non-NAFLD patients.

Further studies with different probes and population cohorts are warranted to corroborate these findings and to understand the underlying mechanism/s responsible for the perturbations in CYP3A4 expression. A well-planned clinical pharmacokinetics investigation would be an ideal study to address the discrepancies in literature but has its limitations where many other factors may contribute to the clearance of probe substrates. Given the fact that our results are primarily limited to Caucasians, care should be taken for interpolation of the results. Therefore, efforts are currently underway in our lab to study different pathways involved in regulation of CYP3A4 expression at protein level.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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List of abbreviations in the order of appearance

BMI	Body mass index
CAR	Constitutive androstane receptor
CPR	NADPH-cytochrome P450 reductase
Cyb5	Cytochrome b5
CYP3A	Cytochrome P450 3A
CYP3A4	Cytochrome P450 3A4
CYP3A5	Cytochrome P450 3A5
CYP3A43	Cytochrome P450 3A43
CYP3A7	Cytochrome P450 3A7
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
HCC	hepatocellular carcinoma
HNF4α	Hepatocyte nuclear factor 4α
HLM	Human liver microsomes
NAFLD	Non-alcoholic fatty liver disease
NAFL	Non-alcoholic fatty liver
NASH	Non-alcoholic steatohepatitis
NADPH	Nicotinamide adenine dinucleotide phosphate
PCR	Polymerase chain reaction
PXR	Pregnane X receptor
TBARS	Thiobarbituric acid reactive substances
TOF	Time of flight

UPLC-MS	Ultra performance liquid chromatography-mass spectrometer

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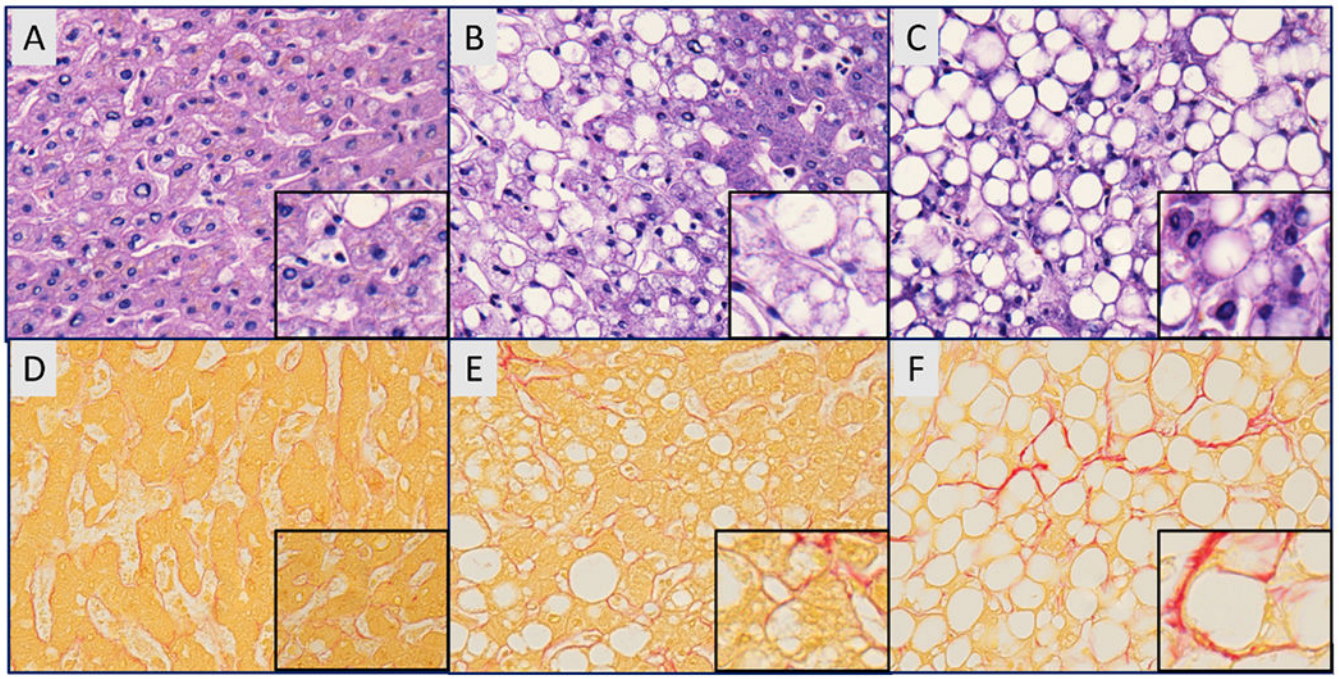

Figure 1.

Adjacent histological sections of liver from (A, D) normal controls, (B, E) patients with diabetes mellitus and hepatic steatosis, i.e. non-alcoholic fatty liver (NAFL), and (C, F) patients with diabetes mellitus and non-alcoholic fatty liver disease with inflammation, i.e. non-alcoholic steatohepatitis (NASH) were stained with (A-C) Hematoxylin and Eosin or (D-F) Sirius Red. (A) Control livers exhibited uniform chord-like arrangements of hepatocytes, (A-Inset) homogeneous cytoplasm, and (D and D-inset) minimal delicate Sirius red staining of sinusoidal collagen. (B) In diabetes, NAFL was associated with (B, B-inset) macrovesicular (large vacuoles filling cytoplasm) and microvesicular (clusters of small cytoplasmic vacuoles) lipid droplets (clear circumscribed structures in cytoplasm) and (E, E-inset) predominantly delicate but focally moderate Sirius red labeling of sinusoidal and pericellular collagen. (C) In patients with diabetes and NASH, the livers showed abundant (C, C-inset) macrovesicular and microvesicular lipid vacuoles in hepatocytes, conspicuous lymphomononuclear inflammatory cell infiltrates among hepatocytes, and (F) prominent Sirus red staining of bridging fibrosis and (F-inset) peri-hepatocyte collagen. (Original magnifications x625)

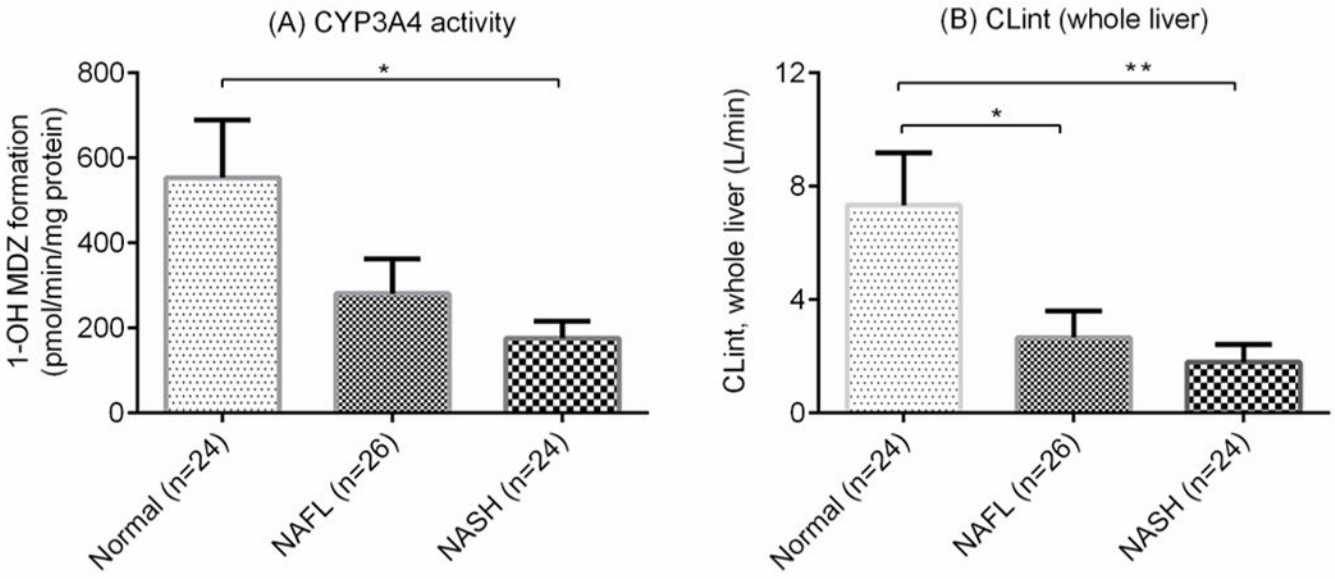

Figure 2. Effect of nonalcoholic fatty liver disease on CYP3A4 activity.

(A) CYP3A4 activity, (B) CLint (whole liver). Column and error bars represent mean±SE. **P*<0.05 as compared to normal. *P*-values reported from nonparametric Kruskal-Wallis test (2-sided) without adjustment for multiple comparisons.

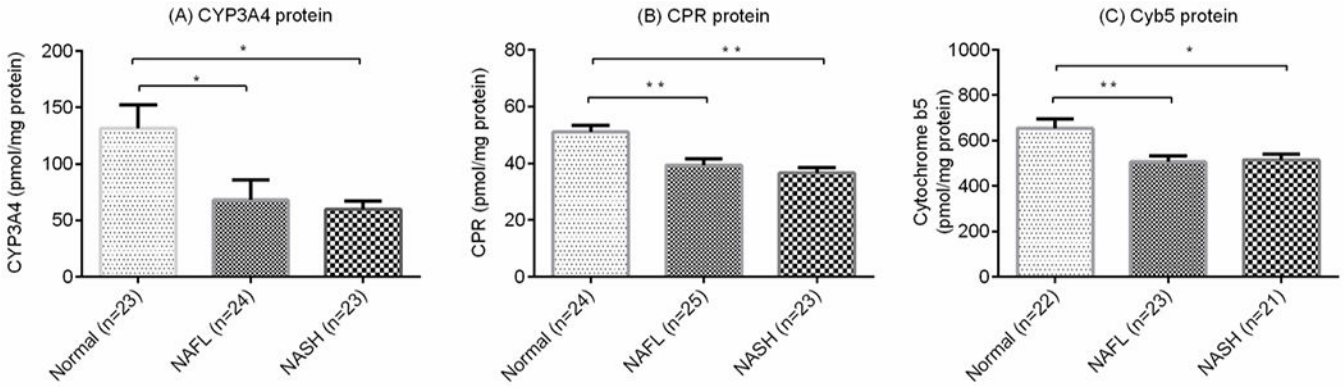

Figure 3. Effect of nonalcoholic fatty liver disease on protein expression.

(A) CYP3A4, (B) cytochrome P450 reductase and (C) Cytochrome b5 protein. Column and error bars represent mean±SE. **P*<0.05 as compared to normal. *P*-values reported from nonparametric Kruskal-Wallis test (2-sided) without adjustment for multiple comparisons.

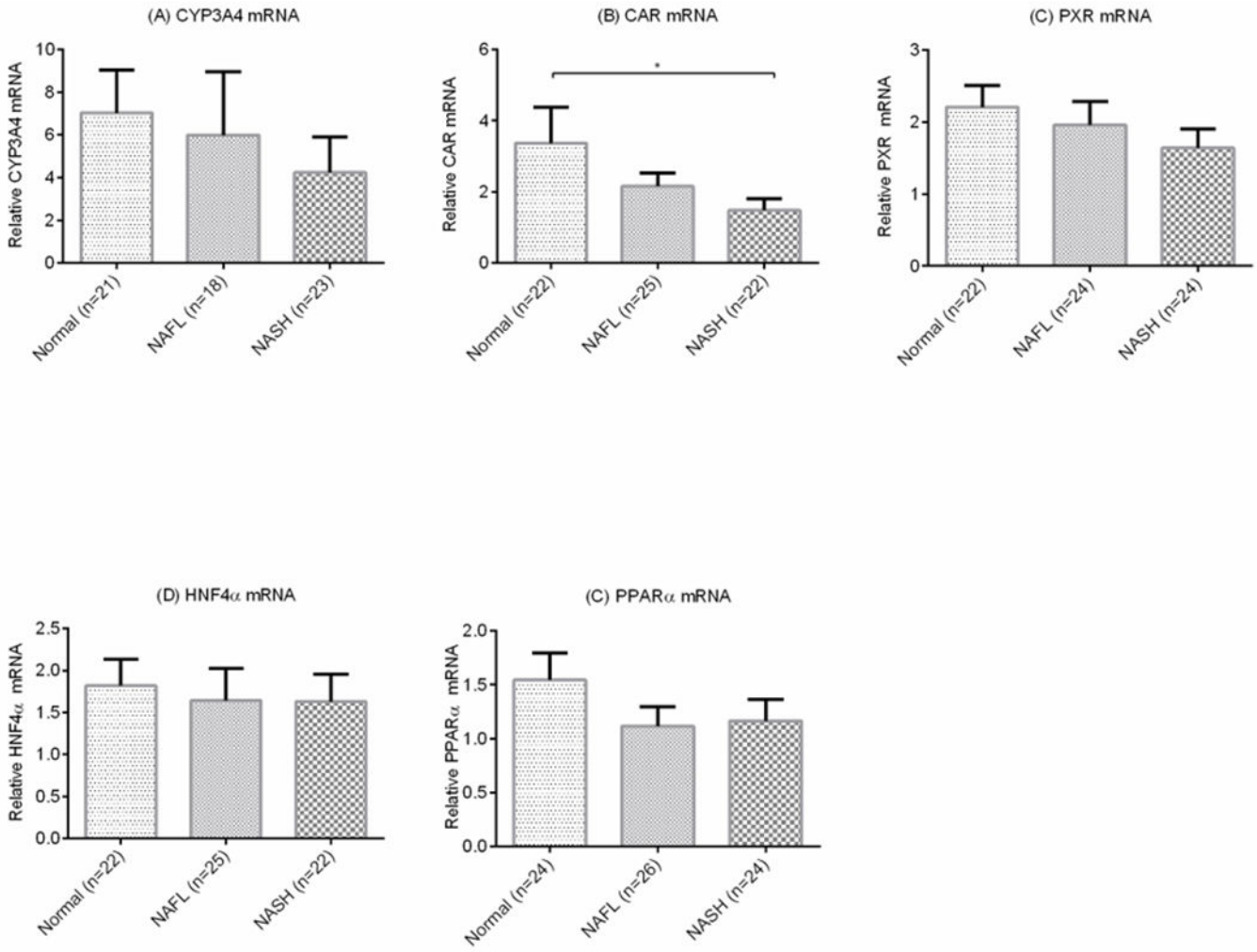

Figure 4. Effect of nonalcoholic fatty liver disease on relative mRNA expression.

(A) CYP3A4 mRNA, (B) CAR mRNA, (C) PXR mRNA and (D) HNF4α mRNA. Column and error bars represent mean±SE. **P*<0.05 as compared to normal. *P*-values reported from nonparametric Kruskal-Wallis test (2-sided) without adjustment for multiple comparisons. Messenger RNA data expressed relative to 18S rRNA

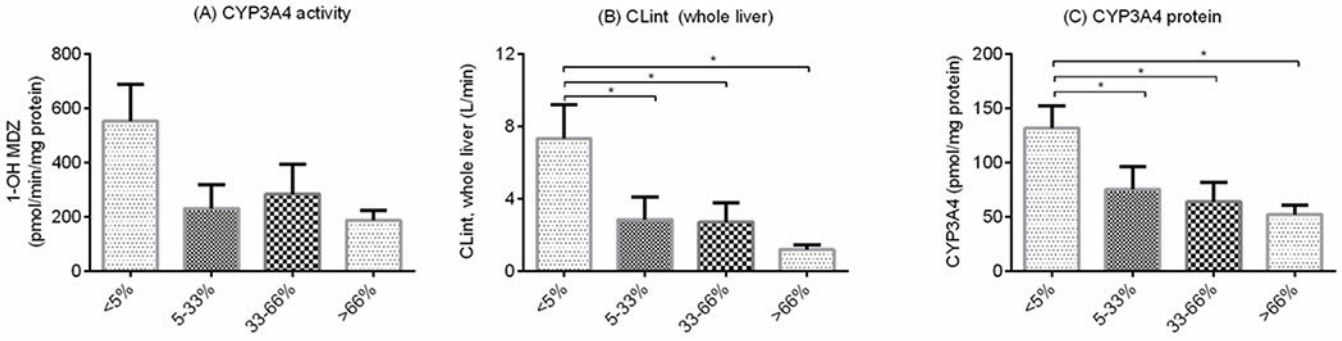

Figure 5. Effect of different grades of steatosis.

(A) CYP3A4 activity, (B) CLint (whole liver), (C) CYP3A4 protein. Column and error bars represent mean±SE. **P*<0.05 as compared to <5% liver fat. *P*-values reported from nonparametric Kruskal-Wallis test (2-sided) without adjustment for multiple comparisons.

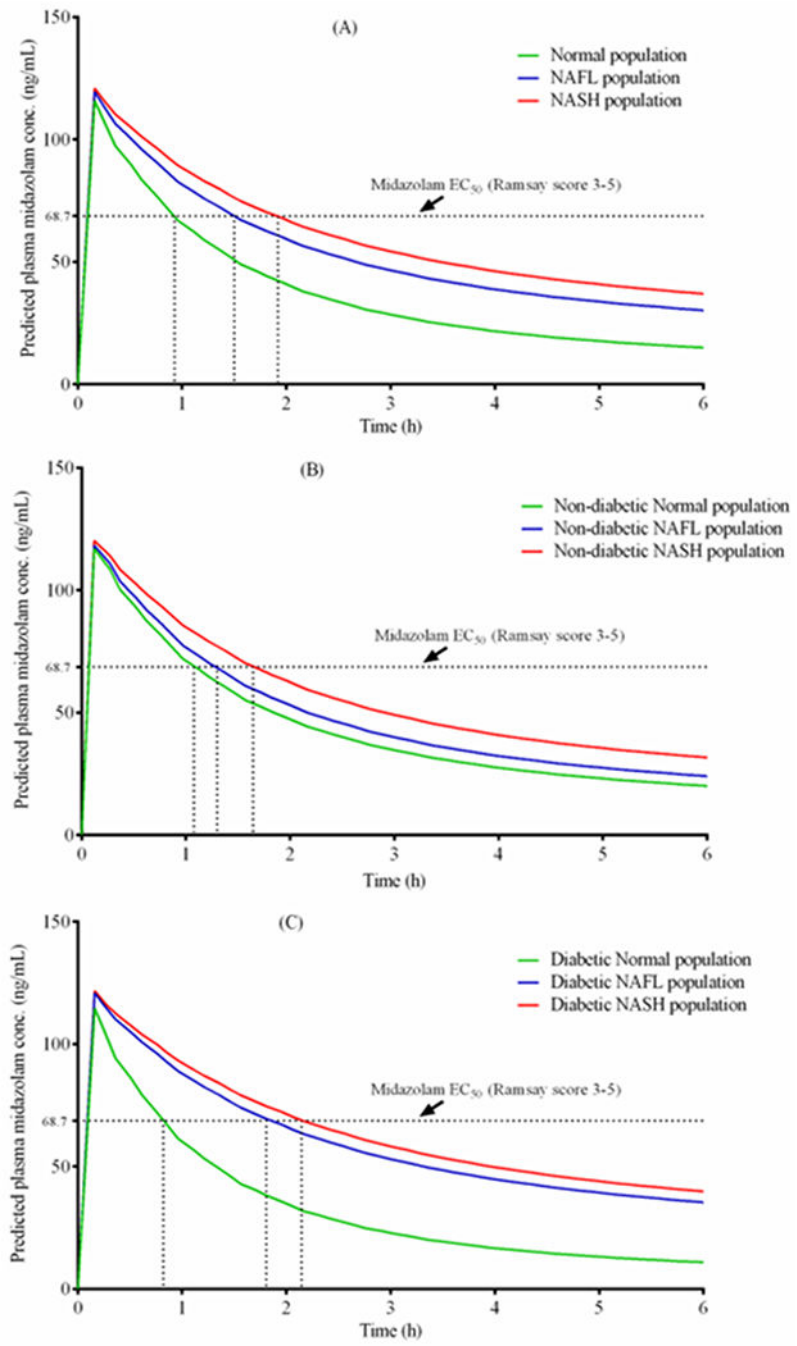

Figure 6. SimCYP predicted plasma concentration of midazolam in virtual Caucasian population indicating a higher concentration and longer sedation time with respect to disease state

(A) in a virtual population of normal, NAFL or NASH patients irrespective of diabetes status (B) without diabetes normal versus NAFL or NASH (C) with diabetes normal versus NAFL or NASH.

Table 1:

Overview of donor demographics

	Normal	NAFL	NASH
n (Male, Female)	24 (13, 11)	26 (10, 16)	24 (11, 13)
Ethnicity[#] (n) C, AA, H	24, 0, 0	26, 0, 0	22, 1, 1
Age[1] (years)	50.2±3.0	52.4± 2.1	53.1±2.1
Body-mass index[1] (kg/m^2)	31.4±3.0	33.9±2.5	32.5±1.6
Diabetes mellitus (no, yes)	11, 13	14, 12	11, 13
Liver weight[1] (kg)	1.6±0.1	2.0±0.2	1.9±0.1
Body weight[1] (kg)	88.7±7.2	97.1±6.6	94.0±4.7
Malondialdehyde [1] (nmol/mg protein)	0.7±0.1	1.2±0.2**	1.5±0.2**
Cholesterol[1] (μg/mg liver)	16.3±1.3	21.0±1.7*	22.6±1.9**
*CYP3A5*3/*3* (n)	24	26	24
*CYP3A4*22* (n) **1/*1, *1/*22*	23, 1	23, 3	23, 1
*CYP3A4*1B* (n) **1/*1, *1/*1B*	24, 0	26, 0	23, 1

[#]C-Caucasian, AA-Afro-American, H-Hispanic;

[1]All descriptive statistics values represent mean±SE.

*$P < 0.05$,

**$P < 0.01$ as compared to normal. *P*-values reported from non-parametric Kruskal-Wallis test (2-sided) without adjustment for multiple comparisons.

Table 2:

Effect of nonalcoholic fatty liver disease on midazolam hydroxylation parameters

	Normal	NAFL	NASH
Vmax (pmol/min/mg protein)	553.8±134.9	281.2±80.9	176.2±40.4 *
Km (μM)	1.6±0.1	2.0±0.2	2.3±0.4
CLint, whole liver (L/min)	7.3±1.8	2.7±0.9 *	1.8±0.6 **

All descriptive statistics values represent mean±SE.

* $P < 0.05$,

** $P < 0.01$ as compared to normal. *P*-values reported from non-parametric Kruskal-Wallis test (2-sided) without adjustment for multiple comparisons.

Table 3:

Effect of nonalcoholic fatty liver disease on protein abundance

	Normal	NAFL	NASH
CYP3A4 (pmol/mg protein)	131.8±20.4	68.0±18.0 *	59.9±7.3 *
CPR (pmol/mg protein)	51.1±2.3	39.3±2.4 **	36.6±1.9 **
Cyb5 (pmol/mg protein)	654.5±41.2	506.5±26.3 *	515.3±24.6 *

All descriptive statistics values represent mean±SE.

* P<0.05,

** *P < 0.01* as compared to normal. *P*-values reported from nonparametric Kruskal-Wallis test (2-sided) without adjustment for multiple comparisons.

Table 4:

Effect of diabetes and NAFLD on CYP3A4 activity, protein and mRNA expression, and relevant proteins and transcription factors

	Non-diabetic			Diabetic		
	Normal (n=11)	**NAFL (n=14)**	**NASH (n=11)**	**Normal (n=13)**	**NAFL (n=12)**	**NASH (n=13)**
Vmax pmol/min/mg protein	528.4±243.7	411.6±140.2	264.2±79.4	575.3±149.8	129.2±34.7**	101.7±17.6**
Km (μM)	1.8±0.2	2.2±0.4	2.6±0.7	1.4±0.1	1.7±0.2	2.0±0.4
CLint (L/min)	5.1±2.6	3.5±1.6	2.8±1.3	9.2±2.6	1.6±0.7**	1.0±0.2**
CYP3A4 (pmol/mg protein)	125.7±30.2	95.1±28.9	75.3±12.2	137.4±28.6	32.8±11.7**	48.1±7.7*
CPR (pmol/mg protein)	48.2±3.16	40.6±3.4	34.0±2.9*	53.5±3.3	37.5±3.4*	38.5±2.4*
Cyb5 (pmol/mg protein)	580.4±42.9	498.6±30.5	495.9±47.7	716.2±62.7	516.6±47.5**	527.3±27.9*
CYP3A4 mRNA	9.5±3.9	8.7±4.7	6.6±3.3	4.8±1.4	1.7±0.9	2.1±0.7
PXR mRNA	2.7±0.5	2.0±0.4	1.7±0.5	3.9±1.8	2.4±0.7	1.3±0.4
CAR mRNA	2.1±0.4	2.2±0.6	1.7±0.4	2.3±0.5	1.7±0.3	1.6±0.3
HNF4α mRNA	1.6±0.5	2.2±0.6	1.9±0.6	2.1±0.4	1.0±0.2*	1.4±0.4
PPARα mRNA	1.6±0.5	1.3±0.3	1.1±0.3	1.5±0.2	0.9±0.2	1.2±0.3

All descriptive statistics value represent mean±SE.

*$P < 0.05$,

*$P < 0.01$ as compared to normal. *P*-values reported from nonparametric Kruskal-Wallis test (2-sided) without adjustment for multiple comparisons. Messenger RNA data expressed relative to 18S rRNA.