Hepatic cytochrome P450 enzyme alterations in humans with progressive stages of non-alcoholic fatty liver disease

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Abbreviations:
P450, cytochrome P450 enzyme; CYP, cytochrome P450 subfamily; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; LTCDS, liver tissue cell distribution system; GAPDH, glyceraldehyde-3-phosphate dehydrogenase, HIF-1α, hypoxia induced factor 1 alpha; TNFα, tumor necrosis factor alpha; IL-1β, interleukin 1 beta
Abstract

Members of the cytochrome P450 enzyme (CYPs) families CYP1, 2 and 3 are responsible for the metabolism of approximately 75% of all clinically relevant drugs. With the increased prevalence of non-alcoholic fatty liver disease (NAFLD), it is likely that patients with this disease represent an emerging population at significant risk for alterations in these important drug metabolizing enzymes. The purpose of this study was to determine whether three progressive stages of human NAFLD alter hepatic CYP expression and activity. Microsomes isolated from human liver samples diagnosed as Normal: \( n = 20 \); Steatosis: \( n = 11 \); NASH (fatty liver): \( n = 10 \), NASH (no longer fatty): \( n = 11 \) were analyzed for CYP mRNA, protein and enzyme activity. Microsomal CYP1A2, 2D6 and 2E1 mRNA levels were decreased with NAFLD progression, while CYP2A6, 2B6 and 2C9 mRNA expression increased. Microsomal protein expression of CYP1A2, 2C19, 2D6, 2E1 and 3A4 tended to decrease with NAFLD progression. Similarly, functional activity assays revealed decreasing trends in CYP1A2 (\( p = 0.001 \)) and 2C19 (\( p = 0.05 \)) enzymatic activity with increasing NAFLD severity. In contrast, activity of CYP2A6 (\( p = 0.001 \)) and CYP2C9 (diclofenac \( p = 0.0001 \); tolbutamide \( p = 0.004 \)) was significantly increased with NAFLD progression. Increased expression of pro-inflammatory cytokines tumor necrosis factor alpha (TNF\( \alpha \)) and interleukin 1 beta (IL-1\( \beta \)) was observed and may be responsible for observed decreases in respective CYP activity. Further, elevated CYP2C9 activity during NAFLD progression correlated with elevated hypoxia induced factor 1 alpha (HIF-1\( \alpha \)) expression in later stages of NAFLD. These results suggest that significant and novel changes occur in hepatic CYP activity during progressive stages of NAFLD.
Introduction

The cytochrome P450 enzyme (CYP) family of heme-containing proteins represents one of the largest and most functionally diverse superfamilies found in nature (Nelson et al., 1993). The main function of CYPs is to facilitate the biotransformation of compounds by addition of functional groups suitable for conjugation and ultimate elimination from the organism (Danielson, 2002). 57 genes and 5 pseudogenes have been identified in the human genome and together these enzymes are responsible for the metabolism of thousands of endogenous and xenobiotic substrates including environmental pollutants, pharmaceuticals, steroids, prostaglandins and fatty acids (Nelson et al., 2004). While P450 expression occurs in a number of organs, including the intestine, lung, kidney and heart (Guengerich, 1994; Kolars et al., 1994; Wheeler and Guenthner, 1990; Zordoky and El-Kadi, 2008), the highest concentration of most CYPs responsible for drug metabolism is in the liver (Krishna and Klotz, 1994; Pelkonen et al., 2008). Members of the CYP1, 2 and 3 families are best known for their crucial involvement in Phase I drug metabolism and account for the biotransformation of approximately 75% of all known therapeutic drugs in humans (Guengerich, 2008; Danielson, 2002). Therefore, much of the research on CYPs has been focused on the regulation, expression and activity of the major drug metabolizing hepatic enzymes in humans, specifically CYP1A2, 2C isoforms, 2D6, 2E1 and 3A4.

Differences in CYP expression along with significant interindividual variation in drug metabolism has been reported in humans. Because of this, it is of utmost importance to fully understand the factors responsible for the regulation of CYPs. In normal human livers, genetic polymorphisms, endocrine imbalance, poor diet, as well as environmental factors can influence the expression of CYPs (Frye et al., 2006; George et al., 1995). Occurrence of one or more of these factors can predispose a patient to altered CYP metabolism and unwanted/negative consequences associated with standard doses of a drug.
Chronic liver disease is another factor that has been reported to impair CYP drug metabolism in patients (Villeneuve and Pichette, 2004). Studies on altered hepatic CYP function have been reported in patients with cholestasis, hepatitis B and C, alcoholic liver disease, and cirrhosis (George et al., 1995; Frye et al., 2006; Tsunedomi et al., 2005; Li et al., 2006; Yang et al., 2003). However, interpretations of the effect of specific diseases have been limited as patients with different types of liver diseases were often placed into a single category. Additionally, in vitro studies of CYP activity in human liver samples from patients with liver disease have yielded conflicting results which have led some to postulate whether regulation of these enzymes may be disease specific (Guengerich and Turvy, 1991; Lown et al., 1992; Lucas et al., 1993). More recently it has been suggested that the severity of liver disease rather than specific disease state correlates with the extent of altered CYP metabolism (Frye et al., 2006).

Non-alcoholic fatty liver disease (NAFLD) is a condition that has received increased attention during the last two decades. Currently, NAFLD is the most prevalent liver disease in the United States, representing 20-30% of all liver disease cases (Bedogni et al., 2005). With obesity and obesity-related conditions (insulin resistance, dyslipidaemia and high blood pressure) identified as predisposing conditions, the occurrence of NAFLD is increasing as well (Targher et al., 2008; Huang et al., 2007). NAFLD composes a spectrum of etiologies ranging from simple fatty liver (steatosis) to the more severe non-alcoholic steatohepatitis (NASH). The proposed mechanism for progression of NAFLD involves a two hit theory where lipid accumulation in hepatocytes (the “first hit”) is followed by a “second hit” including insulin resistance, oxidative stress and cytokine production (Bellentani et al., 2004). Therefore, the goal of the current study was to determine the effect of progressive stages of NAFLD on hepatic CYP expression and function in human tissue.
Materials and Methods

Human liver specimens. Samples of frozen and formalin-fixed, paraffin-embedded adult explant livers [Normal: \(n = 20\); Steatosis: \(n=11\); NASH (fatty liver): \(n=10\), NASH (no longer fatty): \(n = 11\)] were obtained from the Liver Tissue Cell Distribution System (LTCDS) at the University of Minnesota, Virginia Commonwealth University and the University of Pennsylvania. Histological slides were diagnosed using criteria from a scoring system for human NAFLD (Kleiner et al., 2005). Steatotic liver was diagnosed when > 10% of hepatocytes showed fat deposition. NASH with fatty liver diagnosis was defined as having marked inflammation, fibrosis and > 5% of hepatocytes with fat deposition. NASH no longer fatty liver was diagnosed by marked inflammation, fibrosis and < 5% of hepatocytes with fat deposition. Diagnosis was first established by an LTCDS medical pathologist, and confirmed by histological examination at the University of Arizona in a blinded fashion. Information on donors, including age and gender, can be seen in supplemental data-table1.

Total RNA Isolation, and Reverse Transcription. Approximately 50 mg of each human liver sample was homogenized in 3 ml Nucleic Acid Purification Lysis Solution (Applied Biosystems, Foster City, CA). Total RNA was isolated from each sample using the Applied Biosystems 6100 Nucleic Acid Prepstation. For reverse transcription (RT), approximately 200 ng of total RNA from each sample was converted to cDNA following the manufacturer protocol for the Applied Biosystems High Capacity cDNA Archive Kit.

Quantitative RT-PCR (TaqMan®) Analysis. CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, and CYP3A4 cDNA from human liver samples was analyzed using gene-specific TaqMan® primer/probe sets (Applied Biosystems, Foster City, CA). Reactions with the specific primer/probes for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were analyzed as an endogenous control for CYP450 expression. Amplifications
were performed on an ABI 7900HT Real-Time PCR System (Applied Biosystems, Foster City, CA) in relative quantification mode for 40 amplification cycles using standard conditions for TaqMan®-based assays. Threshold cycle (C_T) determinations were performed by the ABI 7900HT system software for both CYP450 and GAPDH gene. Relative-fold mRNA content was determined for each sample relative to the endogenous control gene expression (GAPDH) using the relationship: Relative-fold mRNA Content = 2^{-\Delta\Delta C_T}.

**Human liver microsome isolation.** Human liver samples (~300 mg) were homogenized in 3 ml of buffer A (50.00 mM Tris-HCl, 1.00 mM EDTA and 154.00 mM KCl, pH 7.4) with a dounce homogenizer. Homogenate was centrifuged at 10,000 x g for 30 min at 4°C and the supernatant was collected. Following centrifugation at 100,000 x g for 60 min at 4°C, the supernatant was discarded and the pellet resuspended in 600 µl of buffer B (100.0 mM Sodium pyrophosphate and 0.1 mM EDTA, pH 7.4). Samples in buffer B were centrifuged at 100,000 x g for 60 min at 4°C. The supernatant was resuspended in 300 µl buffer C (10.0 mM KPO_4, 1.0 mM EDTA and 20.0 % glycerol, pH 7.4) and stored at -80°C until analysis.

**Western blot analysis of microsomal CYP levels.** Microsomal protein concentrations were determined using a Bio-Rad protein Assay Reagent Kit (Bio-Rad laboratories, Inc., Hercules, CA) as described by the manufacturer. Microsomal protein levels of P450s and GAPDH (loading control) were determined using a mouse monoclonal antibody specific for human CYP1A2 (Abcam Inc., Cambridge, MA), polyclonal rabbit anti-human CYP2A6, CYP2B6, CYP2C8, CYP2D6, CYP3A4, CYP2E1 (XenoTech LLC, Lenexa, KS), CYP2C9, CYP2C19 (Fitzgerald Industries International Inc., Concord, MA) and a monoclonal rabbit anti-GAPDH antibody (Cell Signaling Technology, Inc., Danvers, MA). Microsomes (10 µg/well) or 10 µg of respective recombinant human CYP protein (BD Gentest from BD biosciences, San Jose, CA) were separated by SDS-PAGE as previously reported (Augustine et al., 2008). Quantification of
relative protein expression was determined using image processing and analysis with Image J in JAVA (NIH, Bethesda, MD) and normalized to respective GAPDH protein expression.

**CYP enzymatic activity determination.** Microsomal activities for human CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1 and CYP3A4/5 were determined using specific marker substrates according to established procedures listed in supplemental data-table 2. Human liver microsomes (~ 0.01-0.04 mg/ml) were incubated at 37°C in potassium phosphate buffer (100 mM, pH 7.4), NADPH (1 mM) and substrate were added to each incubation in methanol or acetonitrile so that the final solvent concentration was 0.1%. Reactions were started by addition of NADPH, and stopped after indicated time points by addition of organic solvent. The amount of product formed was quantified using validated LC-MS/MS methodologies. In each analytical run, at least six calibration standards and 12 quality control (QC) samples (at three different concentration levels) were used to ensure the quality of the analytical run.

**Statistics.** CYP and other expression levels are continuous outcomes with skewed distribution. This asymmetry suggests that median rather than mean values should be compared. Therefore, graphs in this manuscript show box-whisker plots rather than mean values with error bars. The disease groups examined in the current study can be ordered by their severity: normal < steatosis < NASH with fatty liver < NASH no longer fatty. We initially performed two-sample comparisons between each disease state and normal using the Wilcoxon rank-sum test. This is the appropriate alternative to the two-sample t-test when skewed continuous outcomes exist in samples of modest size. For comparing all groups together, we performed a non-parametric test for trend (Cuzick, 1985) rather than using ANOVA, which assumes unordered categories. Thus, instead of simply looking for differences between categories, we used the more
appropriate and logical approach of testing whether expression levels increased or decreased consistently over the ordered disease states. In the results section, NAFLD progression refers to increasing severity of disease state. A significance level of 0.05 was used.

**Immunohistochemical staining of paraffin embedded liver samples.** Formalin-fixed sections of paraffin-embedded livers were deparaffinized in xylenes and rehydrated through a graded alcohol series. Antigen retrieval was performed by incubating slides in citrate buffer (10 mM) for 10 min in a Kenmore 1200 watt microwave set on defrost and endogenous peroxidase activity was blocked with 3% (v/v) H$_2$O$_2$ for 10 min at room temperature. Deparaffinized sections were incubated overnight with either a rabbit polyclonal IL-1β antibody (H-153, Santa Cruz Biotechnology, Santa Cruz, CA), mouse monoclonal TNFα or mouse monoclonal HIF-1α (Abcam Inc., Cambridge, MA) diluted 1:50 in PBS. Protein-antibody complexes were visualized using the Vectastain Elite ABC kit and developed with 3,3’-diaminobenzidine as per manufacturer’s protocol (Vector Laboratories, Burlingame, CA). Negative control staining of human liver sections was performed by incubating without primary antibody.
Results

Histopathology of human livers with progressive stages of NAFLD. Hematoxylin and eosin staining of donor livers was used to assess the severity of NAFLD. Representative images of H&E stained livers from normal, steatotic, NASH with fatty liver and NASH no longer fatty can be seen in Fig 1.

Hepatic CYP mRNA expression during NAFLD progression. There were decreasing trends of CYP1A2 and CYP2C19 mRNA expression associated with progressive stages of NAFLD (p values of 0.225 and 0.193, respectively); however these trends were not statistically significant (Fig 2). CYP2E1 mRNA expression showed a statistically significant decreasing trend (p=0.001) with NAFLD progression. Conversely, CYP2C9 mRNA expression tended to increase with NAFLD progression, but did not reach statistical significance (p=0.220). Similarly, CYP2A6 and CYP2B6 mRNA expression significantly increased with NAFLD progression, with p values of 0.002 and 0.003, respectively. NAFLD progression had little effect on CYP2C8, CYP2D6, or CYP3A4 mRNA expression levels.

Microsomal CYP protein expression in progressive stages of NAFLD. Representative western blots of microsomal CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9/19, CYP2D6, CYP2E1, CYP3A4 and GAPDH are shown in Fig 3. Additionally, relative protein expression of CYPs for all donor samples was determined by densitometry and normalized to GAPDH expression and is seen in Fig 4. Similar to mRNA expression, CYP2A6 protein expression was significantly increased with NAFLD progression (p=0.019). CYP2C8, CYP2D6 and CYP3A4 protein expression tended to decrease with progression of NAFLD (p values of 0.191, 0.068 and 0.112, respectively); however this trend was not statistically significant. CYP1A2 (p=0.0001), CYP2C19 (p=0.01) and CYP2E1 (p=0.01) protein levels significantly decreased with NAFLD progression.
Microsomal CYP enzyme activity during NAFLD progression. Phenacetin O-Dealkylation by CYP1A2 significantly decreased (p=0.001) as the severity of NAFLD increased (Fig 5). Similar to CYP1A2, there was a decreased rate of CYP2C19-mediated mephenytoin 4’-hydroxylation with NAFLD progression (p=0.05). CYP2D6 and CYP3A4 activity toward dextromethorphan and testosterone, respectively, also displayed a decreasing trend with NAFLD progression (p values of 0.062 and 0.18); but these trends did not reach statistical significance. In contrast, CYP2A6 hydroxylation of coumarin was significantly increased with NAFLD progression (p=0.02). Finally, the enzymatic activity of CYP2C9 was determined using two specific substrates of this enzyme. CYP2C9 enzyme activity, determined by diclofenac 4’-hydroxylase and hydroxytolbutamide metabolite formation, was significantly increased with NAFLD progression, with p values of 0.0001 and 0.004, respectively.

Results of two-group comparisons between each disease state and normal. The rank-sum tests did not reveal statistically significant differences between each disease state considered separately vs. normal. Given the modest sample size and the high degree of variability observed in the outcomes, this was not unexpected. However, separate consideration of each disease state discards important information available from the inherent ordering of the disease states. Statistical analysis for trends across ordered categories has the greater power to detect systematic differences than two-sample tests. Thus, the balance of the analyses focused on the use of a non-parametric trend test (Cuzick, 1985) to detect such systematic changes in outcome as a function of NAFLD progression.

Immunohistochemical staining of HIF-1α during NAFLD progression. To determine whether NAFLD induces hypoxia, immunohistochemical staining of donor livers from normal and progressive stages of NAFLD was used to identify expression of known markers, specifically,
HIF-1α. While staining was not observed in normal livers (Fig 6), and only moderate staining was observed in steatotic livers, there was pronounced HIF-1α expression in the cytosol of NASH fatty liver samples and both cytosolic and nuclear staining in NASH no longer fatty liver samples, suggesting that hypoxia occurs in the later stages of NAFLD.

*Immunohistochemical staining of pro-inflammatory cytokines in progressive stages of NAFLD.*

Little to no cytokine staining was observed in normal or steatotic liver tissue (Fig 6). However, there was marked increased expression of TNFα and IL-1β in both stages of NASH, strongly suggesting the presence of inflammation in these stages of NAFLD.
**Discussion**

CYPs have been shown to be particularly susceptible to alterations in expression and activity (Frye et al., 2006). Decreased CYP enzymatic activity can potentially lead to reduced metabolism of therapeutics, ultimately leading to increased bioavailability and possible toxicity. Conversely, increased activity of hepatic CYPs present the potential to increase the metabolism of known substrates thereby decreasing their pharmacotherapeutic effect or increasing the generation of reactive metabolites and oxidative stress. The aim of the current study was to determine whether expression and function of the major drug metabolizing CYPs is altered in human livers diagnosed with progressive stages of NAFLD. To our knowledge, this is the first report of CYP enzyme expression and activity in progressive stages of human NAFLD.

Previous studies have reported up to a 50% decrease in hepatic CYP1A2 protein levels in cirrhotic liver patients when compared to normal liver (George et al., 1995; Congiu et al., 2002). Guengerich et al., noted similar findings in CYP1A2 immunohistochemical staining of livers with sclerosing cholangitis and cirrhosis (Guengerich and Turvy, 1991). CYP1A2 metabolic activity has also been shown to be decreased in primary biliary cirrhosis, alcoholic steatohepatitis and cirrhotic patients as seen by reduced clearance of known substrates antipyrine, theophylline and caffeine (Villeneuve and Pichette, 2004; Lelouet et al., 2001; Bechtel et al., 2000). While we report only a slight downward trend in mRNA expression of CYP1A2, the protein (p=0.0001) and enzyme activity levels (p=0.001) were significantly decreased with NAFLD progression. CYP1A2 has been reported to be significantly decreased in the presence of pro-inflammatory cytokines TNFα and IL-1β (Zhou et al., 2008) and may explain decreased expression and function in the current study.

CYP2A6 plays a role in the metabolism of several clinically relevant drugs, including halothane, disulfiram and valproic acid (Raunio et al., 2001). In the current study we show that mRNA
(p=0.002), protein (0.019) and enzyme activity (p=0.020) of CYP2A6 increased with progressive stages of NAFLD. Significantly elevated levels of CYP2A6 enzymatic activity have been reported in patients with hepatitis, primary biliary cirrhosis as well as alcoholic cirrhosis (Lelouet et al., 2001; Bechtel et al., 2000; Kirby et al., 1996). In addition, induction of CYP2A5, the mouse ortholog of human CYP2A6, has been shown to be induced during oxidative injury to the endoplasmic reticulum as well as during altered redox status (Nichols and Kirby, 2008; Gilmore et al., 2003). It is well documented that oxidative stress occurs in NAFLD patients, as demonstrated by NASH patients who show significantly increased systemic levels of lipid peroxidation products (Chalasani et al., 2004; Videla et al., 2004). It is therefore possible that oxidative stress induced during NAFLD plays a role in the increased CYP2A6 expression and activity reported in this study.

CYP2C9 is generally accepted as the second-most abundant CYP in the human liver and is responsible for the metabolism of a number of clinically relevant drugs substrates including S-warfarin, losartan, rosiglitazone, fluoxetine and tamoxifen (Danielson, 2002). Hepatic CYP2C9 mRNA expression showed an increasing trend with progressive stages of NAFLD (p=0.220); however, there was little overall change in protein expression between samples. Nevertheless, CYP2C9 metabolism of diclofenac was significantly increased with the severity of NAFLD (p=0.0001). In order to verify these results, CYP2C9 enzymatic activity was also determined using a second high affinity substrate, tolbutamide. Similar to diclofenac, hydroxytolbutamide formation by CYP2C9 significantly increased with progressive states of NAFLD (p=0.004).

Several studies have demonstrated that CYP2C9 activity is increased during hypoxia, potentiating metabolism of arachidonic acid into 11,12-epoxyeicosatrienoic acid (Michaelis et al., 2005). 11,12-epoxyeicosatrienoic acid in turn attenuates vascular smooth muscle cell hyperpolarization and resultant vasoconstriction during acute and chronic hypoxic conditions.
(Earley et al., 2003). While no data are available with regard to hypoxia in cases of NAFLD, an experimental model of ethanol induced steatohepatitis in rats showed a significant increase in hypoxia induced factor 1 alpha (HIF-1α) expression in hepatocytes (Li et al., 2006a). In an attempt to explain the observed increase in CYP2C9 activity, we investigated the possibility of hypoxia occurring during NAFLD progression. Fig 6 shows increased cytosolic expression of HIF-1α in NASH with fatty liver samples while both cytosolic expression and nuclear accumulation of HIF-1α was observed in NASH no longer fatty samples. The increased expression and nuclear localization of HIF-1α suggest that hypoxia occurs in the later stages of NAFLD and provides a plausible mechanism for the elevated CYP2C9 activity reported in the current study.

CYP2C19 has been identified as one of the more sensitive P450s to the presence of liver diseases such as hepatocellular carcinoma, hepatitis C and chronic hepatitis and cirrhosis (Frye et al., 2006; Ohnishi et al., 2005). In addition, it has also been shown to be affected earlier than the other important drug metabolizing CYPs (Villeneuve and Pichette, 2004). Results in the current study support these observations as the protein expression (p=0.010) and enzymatic activity (p=0.05) showed statistically significant decreases with progressive states of NAFLD.

Hepatic CYP2C8 mRNA and enzyme activity appeared unaffected by different stages of the disease. It is interesting to note that while CYP2C8 expression and activity remained relatively constant with NAFLD progression, two other relevant members of this subfamily were alternately affected by NAFLD, with increased CYP2C9 activity and decreased CYP2C19 activity. The alternative regulation of CYP2C enzymes observed in this study have not been reported in other liver diseases and warrant further investigation to determine if these changes are specific to NAFLD.
In addition to CYP1A2 and CYP2C19, CYP3A4 regulation and expression have been reported to be particularly sensitive to other liver diseases; however its activity can selectively change with different disease states (Villeneuve and Pichette, 2004). A study by Yang et al in 2003 reported that CYP3A4 activity was significantly altered in patients with cirrhosis, yet remained unchanged by obstructive jaundice (Yang et al., 2003). In the current study CYP3A4 mRNA expression was not different between disease groups, but CYP3A4 protein expression and activity showed decreasing trends with respect to the severity of NAFLD (p=0.112 and p=0.180, respectively). Although those trends did not reach statistical significance, they do seem to indicate that CYP3A4 expression and activity may be decreased with the progression of NAFLD.

CYP2E1 mRNA and protein expression significantly decreased with NAFLD progression, while CYP2E1 metabolism of chlorzoxazone was unaltered by NAFLD progression. The finding that CYP2E1 mRNA and protein decrease during NAFLD conflicts with previously published data which noted increases in livers from patients with NAFLD (Chtioui et al., 2007; Weltman et al., 1998).

In the current study we report that a number of the major hepatic drug metabolizing CYPs are differentially regulated in progressive stages of NAFLD. The expression and activity of CYP1A2, CYP2C19, CYP2D6 and CYP3A4 tended to decrease with increasing severity of NAFLD. However, CYP2A6 and CYP2C9 enzyme activity was significantly increased with progressive stages of NAFLD. With the incidence of NAFLD increasing at an alarming rate, the effect of this disease on major drug metabolizing enzymes is of critical importance. The current study offers a comprehensive analysis of the major hepatic CYP expression and activity in three progressive stages of NAFLD and may provide a valuable framework for physicians when determining the pharmacotherapeutic options and dosing regimens to patients with this disease.
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References


Footnotes

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**Figure Legends**

**Figure 1.** *Histological staining of progressive stages of NAFLD.* Hematoxylin and Eosin stained slides of human liver donor samples were diagnosed histologically using a previously established NAS scoring system. Livers were identified as normal, steatosis (> 10% hepatocyte with fat deposition), NASH with fatty liver (inflammation, fibrosis and > 5% hepatocytes with fat deposition) or NASH no longer fatty (inflammation, fibrosis and < 5% hepatocytes with fat deposition). All representative images are shown at 20x magnification.

**Figure 2.** *Hepatic CYP mRNA content in progressive stages of NAFLD.* Relative mRNA content of hepatic CYPs was determined for all samples (normal, n=20; steatosis, n=11; NASH with fatty liver, n=10; NASH no longer fatty, n=11) by quantitative Real Time-PCR. Relative-fold mRNA content was determined for each sample relative to the endogenous control gene expression (GAPDH) using the relationship: Relative-fold mRNA Content = $2^{-\Delta\Delta C_T}$. Arrows indicate an increasing or decreasing trend in relative mRNA content with respect to NAFLD progression as determined by non-parametric test for trend. Asterisks indicate statistically significant trend as determined by non-parametric test for trend ($p < 0.05$).

**Figure 3.** Representative microsomal CYP protein expression in progressive stages of NAFLD. 10 µg of normal, steatosis, NASH with fatty liver or NASH no longer fatty microsomal protein was resolved by SDS-PAGE. Antibodies specific for respective CYPs or GAPDH were used to identify protein expression of representative individuals from each diagnosis group. Same individuals were used for each CYP.

**Figure 4.** *Quantification of microsomal CYP protein expression in progressive stages of NAFLD.* Following western blot identification of CYP protein levels, densitometry values of all samples...
(normal, n=20; steatosis, n=11; NASH with fatty liver, n=10; NASH no longer fatty, n=11) were normalized to respective GAPDH densitometry values. Arrows indicate an increasing or decreasing trend in protein expression with respect to NAFLD progression as determined by non-parametric test for trend. Asterisks indicate statistically significant trend as determined by non-parametric test for trend ($p < 0.05$).

Figure 5. *Enzymatic activity of microsomal CYPs in progressive stages of NAFLD.* Human microsomes were incubated with specific enzyme substrates. Enzymatic activity was determined by quantification of respective substrate metabolite formation using LC-MS/MS methodologies, data are expressed as % normal liver CYP activity. Arrows indicate an increasing or decreasing trend in protein expression with respect to NAFLD progression as determined by non-parametric test for trend. Asterisks indicate significant trend as determined by non-parametric test for trend ($p < 0.05$).

Figure 6. *HIF-1α, TNFα and IL-1β expression in progressive stages of NAFLD.* Immunohistochemical staining was performed on formalin-fixed paraffin-embedded liver sections from normal, steatosis, NASH with fatty liver and NASH no longer fatty liver. Tissues were counterstained with hematoxylin and images of all groups were taken 40x magnification.
Figure 1

Normal

Steatosis

NASH (fatty liver)

NASH (not fatty)
Fig 2
Figure 3
Fig 4
Fig 6

- **HIF-1α**
  - Normal
  - Steatosis (fatty liver)
  - NASH (not fatty)

- **TNFα**
  - Normal
  - Steatosis (fatty liver)
  - NASH (not fatty)

- **IL-1β**
  - Normal
  - Steatosis (fatty liver)
  - NASH (not fatty)