In Vitro to In Vivo Scalars for Drug Clearance in Non-Alcoholic Fatty Liver and Steatohepatitis

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Running title: IVIVE Scalars in NAFL and NASH

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Number of text pages: 23

Number of tables: 4

Number of figures: 5

Number of references: 41

Number of words in abstract: 250/250 words

Number of words in introduction: 539/750 words

Number of words in discussion: 1470/1500 words

**Abbreviations:** BCA, bicinchoninic acid; BMI, body mass index; CPPGK, cytosolic protein per gram kidney; CPPGL, cytosolic protein per gram liver; GSA, global sensitivity analysis; HomPPGK, homogenate protein per gram kidney; HomPPGL, homogenate protein per gram liver; HLM, human liver microsomes; IVIVE, in vitro-in vivo extrapolation; MPPGK, microsomal protein per gram kidney; MPPGL, microsomal protein per gram liver; NADPH, reduced nicotinamide adenine dinucleotide phosphate; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; PBPK, physiologically based pharmacokinetics; RIN, RNA integrity number; S9PPGK, S9 protein per gram kidney; S9PPGL, S9 protein per gram liver.
Abstract

In vitro-in vivo extrapolation (IVIVE) allows prediction of clinical outcomes across populations from in vitro data using specific scalars tailored to the biological characteristics of each population. This study experimentally determined scalars for patients with varying degrees of non-alcoholic fatty liver disease (NAFLD), ranging from fatty liver to non-alcoholic steatohepatitis (NASH) and cirrhosis. Microsomal, S9 and cytosol fractions were extracted from 36 histologically normal and 66 NAFLD livers (27 NAFL, 13 NASH, and 26 NASH with cirrhosis). Corrected microsomal protein per gram liver (MPPGL) progressively decreased with disease severity (26.8, 27.4, 24.3 mg/g in NAFL, NASH, and NASH/cirrhosis, respectively, compared with 35.6 mg/g in normal livers; ANOVA, p < 0.001). Homogenate, S9 and cytosolic protein showed a consistent trend of decline in NASH/cirrhosis relative to normal control (post-hoc t-test, p < 0.05). No differences across the groups were observed in homogenate, S9, cytosolic and microsomal protein content in matched kidney samples. MPPGL-based scalars that combine protein content with liver size revealed that the reduction in MPPGL in NAFL and NASH was compensated by the reported increase in liver size (relative scalar ratios of 0.96 and 0.99, respectively), which was not the case with NASH/cirrhosis (ratio of 0.63), compared with healthy control. PBPK-informed global sensitivity analysis (GSA) of the relative contribution of IVIVE scalars (hepatic CYP3A4 abundance, MPPGL, liver size) to variability in exposure (AUC) to three CYP3A substrates (alprazolam, midazolam, ibrutinib) revealed enzyme abundance as the most significant parameter, followed by MPPGL, while liver volume was the least impactful factor.
Significance Statement

NAFLD-specific scalars necessary for extrapolation from microsomal or cytosolic in vitro systems to liver tissue are lacking. These scalars are required in clearance prediction and dose selection in NAFL and NASH populations. Previously reported disease-driven changes have focused on cirrhotic livers, with no data on the initial stages of liver disease. We obtained experimental values for microsomal, cytosolic and S9 fractions and assessed the relative impact of microsomal scalars on predicted exposure to different substrate drugs using physiologically based pharmacokinetics.
Introduction

Non-alcoholic fatty liver disease (NAFLD) represents a significant global health concern, affecting approximately 30% of the worldwide population, with a clinical spectrum extending from simple steatosis to non-alcoholic steatohepatitis (NASH) (Younossi et al., 2023). NASH encompasses liver inflammation, hepatocyte damage, and fibrosis, elevating the risk of progression to cirrhosis and hepatocellular carcinoma (Rinella, 2015; Marjot et al., 2020). Apart from these pathological modifications, various liver conditions associated with NAFLD influence drug kinetics and therapeutic outcomes by altering the expression and function of drug-metabolizing enzymes (DMEs), potentially affecting efficacy or causing adverse reactions (Merrell and Cherrington, 2011; Cobbina and Akhlaghi, 2017). Despite the widespread prevalence of NAFLD, limitations in diagnostic methods and the absence of precise biomarkers and comprehensive models hinder the development of novel medications (Murphy et al., 2023). Exploring in vitro pharmacokinetic systems for NAFLD, this study highlights the need for improved translational methods and emphasizes the ongoing challenges in understanding and managing this complex liver condition.

Physiologically based pharmacokinetic (PBPK) modeling enables prediction of changes in drug exposure and effects for patients from special populations lacking drug label information (Darwich et al., 2021). Extrapolation to hepatic clearance (IVIVE) within a PBPK framework requires scaling drug data measured in relevant in vitro systems, such as recombinant enzymes, hepatocytes, liver microsomes, and cytosol. In IVIVE, scaling from enriched subcellular fractions is standard practice, relying on robust estimates of relevant scalars, notably the protein content from the specific tissue and the abundance of rate-limiting enzymes (Neuhoff et al., 2021; Rostami-Hodjegan, 2012). Accounting for these protein contents quantitatively is crucial to ensure accurate scalar values are used for improved clearance and exposure predictions using PBPK models (Leeder et al., 2022). In the case of microsomal protein per gram liver (MPPGL) employed for scaling in vitro intrinsic clearance measured in microsomes, protein content is used in conjunction with protein loss estimation with different markers, such as NADPH.
cytochrome P450 reductase or total P450 content. Mean MPPGL in healthy human liver tends to range between 32 and 40 mg/g liver, as established previously (Barter et al., 2007), while other scalars for cytosolic or S9 fractions are not well established.

Variations in MPPGL due to demographic or clinical factors, such as disease and age, have been explored, with differences in marker specificities contributing additional variability in reported values (Barter et al., 2007, 2008). In the case of disease, whereas scaling factors have been reported in human liver samples obtained from cirrhosis populations with different etiologies (El-Khateeb et al., 2020), data are still lacking for NAFL and NASH, as highlighted in a recent gap analysis (Murphy et al., 2023). The aim of this study was to derive scaling factors in liver tissue from patients with NAFL, NASH and NASH with cirrhosis, as progressive disease stages, focusing on comparison of these metrics with those from healthy or histologically normal tissues. The work underscores the necessity of using population-specific scalars for accurate drug pharmacokinetic predictions in NAFL and NASH. The importance of the MPPGL scalar was assessed with global sensitivity analyses (GSA) by simulation of CYP3A substrate drugs with different extraction ratios to assess impact on systemic exposure. The study presents novel experimental data with the ultimate aim of addressing dosage adjustment in NAFL and NASH populations.

**Materials and Methods**

**Tissue samples and study design**

Human specimen sets used in this study were either surgical liver samples or post-mortem matched liver and kidney cortex samples supplied by University of Kansas Medical Centre, Kansas City, KS, and Amsbio LLC, Cambridge, MA, respectively. Samples were collected with prior informed consent from the donors under ethical approval (CR00011831 and PG-ONC2003/2, respectively). Donors were adults previously diagnosed with NAFLD and the samples were categorized by severity of disease (NAFL, n = 27; NASH, n = 13; cirrhosis, n = 26). The cirrhosis samples (n = 26) were classed using their Child-Pugh scores to class A (n = 2), B (n = 7) and C (n = 17). Post-collection histopathology
examination of the tissue confirmed the diagnosis. A set of samples from normal adult livers (n = 36), obtained either post-mortem or surgically from patients with non-metastatic liver disease, was used as control. The post-mortem tissue samples (post-mortem interval range: 1.5–5.0 h) were of high quality with average RNA integrity number (RIN) of >7/10 for both liver and kidney sets (range: 6 to >8). A summary of the demographic and clinical information is provided in Table 1. Detailed demographic and clinical information is listed in Supplemental Tables 1 and 2.

**Tissue homogenization and fractionation**

Unless otherwise stated, all chemicals were purchased from Sigma-Aldrich (Burlington, MA). Tissue samples were processed as previously reported (El-Khateeb et al., 2020; Vasilogianni et al., 2021), with minor modifications. Fresh-frozen liver and kidney tissues were weighed (range: 113–180 mg), minced into small pieces with surgical scalpels, and placed in 2 ml tubes with 1.4 mm ceramic beads (Omni International, Kennesaw, GA). A Bead Ruptor 24 homogenizer (Omni International, Kennesaw, GA) was used for homogenization in a buffer containing 150 mM KCl, 2 mM EDTA, 50 mM Tris, 1 mM dithiothreitol, and EDTA-free protease inhibitor cocktail (pH 7.4), at 10 ml homogenization buffer per gram tissue. Homogenization was performed in four cycles, each cycle consisting of 10 seconds at 6.3 m/s with 5-minute intervals on ice. After homogenization, sonication was performed for 10 seconds using an ultrasonic 1/8” probe at 50% amplification (VWR, Randor, PA) on ice to break down any remaining cells in suspension. The resulting homogenate was then centrifuged at 10,000 g for 20 minutes at 4°C using a 5424R centrifuge (Eppendorf, Hamburg, Germany). The S9 fraction (supernatant) was recovered and subjected to ultra-centrifugation at 100,000 g for 75 minutes at 4°C using an Optima MAX-TL ultracentrifuge (Beckman Coulter, Indianapolis, IN). The resulting supernatant (cytosol) was stored at -80°C, while the pellet (microsomes) was resuspended in 1 ml of storage buffer (0.25 M potassium phosphate, pH 7.25) per gram of liver/kidney tissue and stored at -80°C.

**Protein content measurement**
The protein content of the homogenates, S9 fractions, cytosolic fractions, and microsomal fractions was measured using a bicinchoninic acid (BCA) protein assay kit (EMD Millipore, Billerica, MA), following the manufacturer’s instructions and using bovine serum albumin as a standard (Thermo Fisher Scientific, Waltham, MA).

**Measurement of cytochrome P450 reductase activity**

To assess recovery and enrichment of reticular proteins (including cytochrome P450 enzymes and glucuronosyltransferases) following fractionation, cytochrome P450 reductase activity was measured as a marker of reticular membrane (Guengerich et al., 2009; Achour et al., 2011). The assay (Tribioscience, Sunnyvale, CA) was conducted following the manufacturer’s instructions with some modifications. Briefly, the amount of protein from each fraction (homogenate, S9, cytosol or microsomal fractions) originating from 0.25 mg of liver tissue was added to 140 µl of assay buffer and 5 µl of equine cytochrome c in a 96-well plate. Baseline absorbance at 550 nm was measured every 5 seconds for 2 minutes by a SpectraMax Plus 384 UV-Visible spectrophotometer (Molecular Devices, San Jose, CA) operated in kinetic mode. The reaction was initiated by adding 5 µl of 20 mM reduced nicotinamide adenine dinucleotide phosphate (NADPH), and the absorbance was recorded every 5 seconds for 10 minutes. The assay was applied in the same manner to fractions from individual liver samples (n = 101) and a pool of all healthy liver samples (n = 36), measured in triplicate.

**Data processing**

Protein content data above the limit of quantification were accepted for further analysis. The lower limit of quantification was established based on the protein assay’s calibration curve for the homogenate (28.1 mg/g tissue), S9 (25.9 mg/g tissue), cytosol (24.6 mg/g tissue) and microsomes (4.0 mg/g tissue). The ratio of the slopes of the initial linear phase (determined visually) of cytochrome P450 reductase activity (Fig S1) measured in S9, cytosolic and microsomal fractions relative to the corresponding homogenate from each liver sample was used to assess the recovery of reticular proteins in S9, cytosol and microsomes, respectively (Eq 1 and 2).
Activity (U/mg liver) = \frac{(\Delta A_{550} / \text{min}) \times \text{dil} \times \text{reaction volume}}{21.1 \times \text{enzyme volume} \times \text{conversion factor}} (1)

Recovery factor = \frac{\text{Reducatase activity in fraction (U/mg liver)}}{\text{Reducatase activity in homogenate (U/mg liver)}} (2)

Where cytochrome P450 reductase activity is measured in U/mg liver such that 1 U will reduce 1 µmol of oxidized cytochrome c per minute at pH 7.8 at 25°C; \( \Delta A_{550} / \text{min} \) is the rate of change in absorbance at 550 nm = (\( \Delta A_{550\ _\text{sample}} \) – \( \Delta A_{550\ _\text{blank}} \)); dil is the dilution factor of the original enzyme sample (1:10); enzyme volume is the volume in ml of the sample equivalent to 0.25 mg of tissue, and 21.1 is the extinction coefficient for reduced cytochrome c. The reaction volume is the total volume of the assay (0.2 ml). The conversion factor converts the activity from units of U/ml to U/mg tissue and is specific to each sample (ratio of protein concentration in the diluted fraction to the amount of fractional protein per mg of tissue in units of mg/ml). For all samples, enzyme volume \times \text{conversion factor} was equivalent to 0.25 mg tissue.

The recovery ratio for microsomes relative to homogenates was used to correct the measured microsomal protein per gram liver (MPPGL) for each liver sample (Eq 3).

\text{Corrected MPPGL} = \frac{\text{Microsomal protein content (mg/g liver)}}{\text{Recovery factor}} (3)

Enrichment of reticular proteins in S9 and microsomal fractions was measured using Eq 4 for fractions and homogenates from the same tissue.

\text{Enrichment} = \frac{\text{Reducatase activity per unit (µg) protein in fraction}}{\text{Reducatase activity per unit (µg) protein in homogenate}} (4)

\textbf{Statistical analysis}

Statistical analysis aimed to assess differences between scalars in disease groups relative to healthy control. The data were assessed for normality of distribution using the D’Agostino-Pearson and the Kolmogorov-Smirnov normality tests. Overall, the distribution of data was normal; one-way analysis of
variance (ANOVA), followed by pairwise \( t \)-tests were used to assess differences between the disease datasets. Differences between sample sets (surgical and post-mortem), male and female donors, age groups and BMI groups were assessed using ANOVA or \( t \)-tests, depending on the number of compared sets. Correlations between liver and kidney data were assessed using Spearman’s correlation test. Statistical analysis was carried out using Excel 365 and GraphPad Prism 10.0.2 (GraphPad Software, La Jolla, CA).

**Global sensitivity analysis of the effect of scalars on simulated drug exposure**

The impact of altered MPPGL in combination with hepatic CYP3A abundance and liver size on exposure to alprazolam (0.5 mg PO), midazolam (5 mg PO) and ibrutinib (140 mg PO) (CYP3A substrates with different hepatic extraction) was investigated by global sensitivity analysis (GSA) implemented in Simcyp® Version 22 Release 1 (Certara, Princeton, NJ) using available population models in the Simcyp® library. The primary objective was to assess the relative importance of the three hepatic scaling factors (enzyme abundance, MPPGL and liver size) used to extrapolate intrinsic clearance to in vivo hepatic clearance. In addition, we also considered other relevant systems parameters (which are important for assessing systemic drug exposure but are not considered hepatic scalars), including human serum albumin, intestinal CYP3A4, and hepatic blood flow. The literature was analyzed for physiologically plausible boundaries derived from data pertaining to both healthy and diseased subjects.

Adult MPPGL from the literature and the present study varied between 8.2 and 74.3 mg protein per gram liver in 154 predominantly White individuals aged 18 to 96 years (El-Khateeb et al., 2020; Vasilogianni et al., 2021, 2022). The abundance of CYP3A in Caucasian adult livers ranged from below the limit of quantification to 601 pmol/mg microsomal protein in 713 livers for CYP3A4 and to 291 pmol/mg protein in 250 livers for CYP3A5 (Achour et al., 2014). Total liver volume ranged from 0.8 to 2.7 L in >2,000 adult subjects (Li et al., 2003; Johnson et al., 2005; Bian et al., 2015; Ozaki et al., 2016). Hepatic blood flow in 98 subjects aged 18 to 78 years ranged from 545 to 3136 ml/min (Huet et
al., 1975; Zoli et al., 1995; Jakab et al., 1996; Yzet et al., 2010). The levels of CYP3A4 in the small intestine in 53 adults aged 17 to 80 years varied between 5 and 37 pmol/mg protein (Couto et al., 2020; Wegler et al., 2022). Additionally, human serum albumin levels ranged from 30 to 54 g/L (Alrubia et al., 2022). A summary of the systems parameters used in GSA can be found in Supplemental Table 5. Direct assessments were conducted for the ranges of MPPGL, hepatic CYP3A4 and CYP3A5, intestinal CYP3A4, and serum albumin. The determination of liver volume was integrated by varying body surface area, consistent with equations reported by (Johnson et al., 2005). Cardiac output was used as a surrogate to assess the effect of variability in hepatic blood flow (at a fixed percentage of cardiac output).

Simulations were conducted using a virtual Northern European Caucasian population (10 trials x 10 male subjects, aged 18–95 years). The simulations focused exclusively on adult males to simplify physiological data as the study did not necessitate discerning differences in drug pharmacokinetics between sexes or in pediatric populations. The frequency was uniform for all parameters, and the simulations were carried out using minimal PBPK models for the three drugs. The Morris method was applied, which defines the impact of a parameter by their influence on the output variable. The impact of variability in tested parameters on AUC (as a measure of exposure) was investigated, and the relative contribution of each parameter was reported as a percentage of total absolute mean AUC.

Results

In this study, homogenate, S9, cytosol and microsomal protein content was measured in samples from healthy livers (control, n = 36) and different NAFLD progression stages (NAFL, n = 27; NASH, n = 13; and cirrhosis, n = 26). In addition, a subset of liver samples had matching kidney samples (normal, n = 17, NAFL, n = 7; NASH, n = 7). The samples were processed to generate a set of IVIVE scaling factors for liver and kidney.

Protein content in liver and kidney fractions from control and liver disease samples
One matched post-mortem liver and kidney sample (sample ID: 091660A and 053081A, respectively) from the NASH subset yielded green-colored homogenate, which did not produce a recoverable pellet upon ultra-centrifugation; the matched sample was therefore excluded from further processing. Scalar data from liver (n = 101) and kidney (n = 30) sample sets (surgical and post-mortem) are summarized in Tables 2 and 3, respectively (details are listed in Supplemental Tables 3 and 4, respectively). Samples that yielded values lower than the limit of quantification were excluded from analysis. Collated hepatic scalar data are compared in Fig 1.

Mean homogenate protein per gram liver (HomPPGL) was significantly different across liver tissue groups (Fig 1a, ANOVA, p < 0.001). HomPPGL values in the cohort with NASH and cirrhosis were significantly lower (115.0 ± 16.0 mg/g) compared with normal control (136.7 ± 23.4 mg/g), NAFL (139.4 ± 19.8 mg/g), and NASH (141.9 ± 19.2 mg/g) groups (t-tests, p < 0.001). There were no significant differences in mean S9 protein per gram liver (S9PPGL) or mean cytosolic protein per gram liver (CPPGL) among the liver groups (ANOVA). However, pairwise t-tests showed significantly lower (p < 0.05) mean S9PPGL in the NASH/cirrhosis group (84.9 ± 17.4 mg/g) compared with the normal (95.4 ± 19.9 mg/g) and NAFL (98.2 ± 25.6 mg/g) sets. Significantly lower (p < 0.01) mean CPPGL values (59.9 ± 11.6 mg/g) in NASH/cirrhosis were also observed compared with normal control (68.6 ± 13.1 mg/g).

No difference in homogenate protein per gram kidney (HomPPGK), S9 protein per gram kidney (S9PPGK), cytosolic protein per gram kidney (CPPGK), or microsomal protein per gram kidney (MPPGK) in kidney tissue from NAFL and NASH groups compared with the control group (Fig 2).

Recovery and enrichment of hepatic reticular proteins in microsomal and S9 fractions

Cytochrome P450 reductase activity was used as a marker of hepatic reticular membrane protein (Fig S1). Activity was measured in homogenates, S9, and microsomal fractions (Supplemental Table 3). The slopes were calculated twice independently by the two authors. Mean activity in homogenates in the control, NAFL, NASH, and NASH/cirrhosis groups was 2.29 (± 1.48), 2.92 (± 1.05), 1.43 (± 0.99), and 2.26 (± 0.88) nmol/min/mg liver, respectively. Mean activity in S9 fractions was 2.08 (± 1.43), 2.29
(± 0.91), 1.24 (± 0.77), and 2.09 (± 0.83) nmol/min/mg liver, respectively. Mean activity in microsomal fractions was 1.60 (± 1.18), 1.82 (± 0.69), 1.04 (± 0.68), and 1.90 (± 0.79) nmol/min/mg tissue, respectively. Mean recovery and enrichment data are shown in Fig 1b, 1c and Table 2, and individual values are listed in Supplemental Table 3. Recovery values in liver S9 fractions ranged from 38.5% to 100% (mean: 86.2%) in the set of individual samples (n = 92), whereas the range of recovery in liver microsomes was 25.2%–100% (mean: 70.8%). Recovery of reticular proteins in cytosol was negligible (13.5% in a pool of 36 healthy livers). Average enrichment in liver S9 and microsomes was 1.2- and 5.0-fold, respectively (n = 92).

**Effect of progressive liver disease on microsomal protein per gram liver (MPPGL)**

The microsomal recovery values (Fig 1b) allowed correction of the liver microsomal scalars (Table 2). A summary of the corrected MPPGL values is shown in Fig 1d. Mean corrected MPPGL was 35.6 ± 9.9 mg/g in normal livers, 26.8 ± 12.2 mg/g in NAFL, 27.4 ± 6.6 mg/g in NASH, and 24.3 ± 5.8 mg/g in NASH with cirrhosis. There was a progressive decline in MPPGL with disease severity; ANOVA test showed a significant difference across the four groups (p < 0.001) and post-hoc pairwise t-tests showed MPPGL values were significantly lower in NAFL (p < 0.01), NASH (p < 0.05), and NASH/cirrhosis (p < 0.001) compared with normal levels.

**Covariates of hepatic and renal scaling factors**

The protein content of homogenate, S9, cytosol, and microsomal fractions was measured in liver and kidney tissue. Values were higher in liver than kidney (Fig S2), and rank-order correlation between protein content in post-mortem liver and kidney was moderate in homogenate, S9, and cytosol fractions, while no correlation was observed for microsomal fractions (Fig S3). For the post-mortem samples, the post-mortem interval up to 5 hours did not lead to considerable changes in the protein content in tissue fractions from liver or kidney (Fig S4). Recovery (S9 and microsomal), enrichment (S9 and microsomal), and protein content were compared between post-mortem and surgical liver samples, indicating higher protein recovery and comparable enrichment in S9 and microsomal fractions from
surgical samples (Fig S5). The protein content in post-mortem and surgical samples was comparable in homogenates and cytosolic fractions, whereas S9 content was slightly higher (1.16 fold; \( t \)-test, \( p < 0.05 \)) and microsomal content was slightly lower (0.87 fold; \( t \)-test, \( p > 0.05 \)) in surgical samples compared with post-mortem liver fractions.

Unlike liver disease (Fig 1), other donor characteristics (sex, age after maturity and BMI) had no significant impact on protein content of homogenate, S9, cytosol and microsomal fractions from liver and kidney (\( t \)-test and ANOVA, \( p > 0.05 \)) (Fig 3 and Fig 4) across the different sample sets. The BMI of the four groups of donors was very similar, with an average BMI of 30-32 kg/m\(^2\), indicating the majority of the donors were in the overweight/obese range (Fig 3c). The effect of ethnicity on liver and kidney scaling factors could not be determined because the subjects were predominately of the same ethnicity (White).

**Comparison of measured scalars with available scaling methods**

The current study utilized experimental MPPGL-based scaling methods with total liver volume for disease populations relative to control (Table 4). The method was applied by multiplying the average measured microsomal protein content per gram liver by reported mean total liver volume for each cohort (Bian et al., 2015; Ozaki et al., 2016). The measured scalars for NASH and NAFL populations, reported here for the first time, were comparable to levels in a healthy population (0.96 and 0.99 fold difference, respectively). The fold difference in scalars relative to healthy control mirrored the same trend of decline reported previously (Johnson et al., 2010; El-Khateeb et al., 2020; Simcyp (Certara) meta-analyses for versions 18/19 and 20-22) from healthy to progressive cirrhosis. The results were similar to previously reported MPPGL-based scalars across the stages of cirrhosis (El-Khateeb et al., 2020). Empirical scaling methods (Johnson et al., 2010; Simcyp (Certara) meta-analyses), which utilize functional liver volume of the disease cohort, also generated scalars comparable to MPPGL-based scalars in this study in mild to severe stages of cirrhosis.

**Global sensitivity analysis of the effect of scalars on simulated drug exposure**
Global sensitivity analysis was conducted to assess the relative influence of key system parameters employed in scaling of in vitro data to in vivo outcomes. These parameters included MPPGL, liver size and abundance of hepatic CYP3A, alongside other pertinent factors, including human serum albumin, intestinal CYP3A4, and hepatic blood flow, with a focus on their impact on exposure (AUC) to three CYP3A substrates (Fig 5). The analysis was intended to assess the relative importance of MPPGL toward simulated variability in AUC compared with other hepatic scalars.

As expected, abundance of hepatic CYP3A4, the rate-limiting drug-metabolizing enzyme for the three substrates, had the highest impact on exposure (28-51% of variability in AUC). MPPGL emerged as the second most influential factor, representing 17-32% of output variability. Abundance of hepatic CYP3A5 contributed to 13-33% of variability, whereas liver volume accounted for 10-16%, serum albumin contributed 5-6%, hepatic blood flow contributed up to 9%, and the abundance of CYP3A4 in the small intestine contributed up to 4% of output variability. MPPGL together with liver size (changes in the combined parameter in different cohorts are shown in Table 4) contributed 28-48% of overall variability in AUC, which is close to the size of the impact of hepatic CYP3A4 abundance.

**Discussion**

NAFL leading to NASH, is a progressive liver disease that may impact drug and xenobiotic disposition as reported in small sample size studies (Murphy *et al.*, 2023). This condition can also end in liver cirrhosis, with demonstrated changes in MPPGL among other systems parameters, possibly leading to differences in metabolic and transport clearance (El-Khateeb *et al.*, 2020, 2021a). Importantly, the impact of changes in scaling factors, particularly MPPGL, on metabolic clearance in NAFL and NASH patients remains unexplored (Murphy *et al.*, 2023). Given the progressive nature of NAFLD —spanning from NAFL to NASH and ultimately NASH with cirrhosis— the effects in each disease stage necessitate sufficient examination. Therefore, this study aimed to explore, for the first time, how the different progressive stages of NAFLD influence a range of liver and kidney IVIVE scalars.
MPPGL values were determined across different NAFLD cohorts, with values in histologically normal tissues aligning with reported data (35.6 ± 9.9 mg/g liver compared with reported MPPGL of 34 mg/g liver from collated data (Barter et al., 2007)). In NAFL and NASH, MPPGL decreased moderately with disease severity (26.8 ± 12.2 mg/g in NAFL, 27.4 ± 6.6 mg/g in NASH, and 24.3 ± 5.8 mg/g in NASH with cirrhosis), which may reflect a global reduction in microsomal protein content per unit tissue mass as fat deposits in tissue become more significant. This decline may also lead to decreased abundance of microsomal proteins (pmol/g liver), such as hepatic enzymes and transporters (Murphy et al., 2023).

HomPPGL also exhibited significant differences across liver tissue groups, driven mainly by the lower values in NASH with cirrhosis (115.0 ± 16.0 mg/g) compared with normal levels (136.7 ± 23.4 mg/g), as well as those in NAFL (139.4 ± 19.8 mg/g) and NASH (141.9 ± 19.2 mg/g). Likewise, S9 and cytosolic protein content was significantly lower in the NASH/cirrhosis group compared with normal control, indicating a common trend of difference between cirrhotic and healthy livers.

By contrast, HomPPGK, S9PPGK, CPPGK, and MPPGK measured in matching kidneys remained consistent across the control and liver disease groups. Investigating the effects of NAFLD on renal elimination capacity is of interest to modelling renally cleared drugs, considering that the mechanisms at play in NAFLD, including inflammation, oxidative stress, and fibrogenesis, may contribute to kidney injury, although the exact mechanism is still unclear (Heda et al., 2021). The data reported in this study, for the first time, indicate that liver disease did not exert any discernible impact on fractional protein content in kidneys, albeit in a small number of kidney samples.

The prevalence of NAFL and NASH has been contextually reported in relation to different age, sex, BMI, and ethnicity groups. In the United States, NAFLD is more commonly found in young to middle-aged men, post-menopausal women, those with a BMI of 30 kg/m² or higher, and among individuals of Hispanic ethnicity (Marjot et al., 2020). Assessment of differences in demographics between the healthy cohort and disease groups is beyond the scope of this study due to the small sample size. Notably, disease remained the most important determinant of liver protein content, and for all groups,
donor characteristics, such as age, BMI, and sex, had little impact on protein content. Results for age and sex in the cirrhosis group aligned with previously reported data (El-Khateeb et al., 2020).

The changes in MPPGL values described herein cannot be considered in isolation from changes in liver volume due to NAFLD, reported to increase in NAFL and NASH, and then decline in cirrhosis (Bian et al., 2015; Ozaki et al., 2016). The current study utilized experimental MPPGL scaled with total liver volume in disease populations relative to control. We report, for the first time, MPPGL-based scaling parameters for NAFL and NASH populations, and we add confirmatory scaling data in relation to different grades of cirrhosis with NASH. Scaling data to predict hepatic clearance in populations with cirrhosis, explored in this comparison, relied either on empirical parameters related to functional liver volume or MPPGL-based scalars combined with total liver volume (El-Khateeb et al., 2020). In contrast to MPPGL-based scalars, empirical parameters are determined through measurement of radioligand binding to viable hepatic cells and computed tomography image processing (Matsui et al., 1996; Miki et al., 2001), relying on the assumption of comparable microsomal protein yields in functional cells of diseased and healthy livers, based on the intact cell theory (McLean and Morgan, 1991). This assumption does not account for variations in total liver size between cirrhotic and healthy livers, rendering the method an indirect approach to estimating scaling factors for different subcategories of cirrhosis (El-Khateeb et al., 2020).

The measured scalars for NASH and NAFL populations were comparable to those in a healthy population (0.96- and 0.99-fold difference, respectively). The fold difference in scalars relative to healthy control mirrored the declining trend reported previously (Johnson et al., 2010; El-Khateeb et al., 2020) from healthy to progressive cirrhosis. Whereas the results were similar compared with previous MPPGL-based scalars in mild cirrhosis (El-Khateeb et al., 2020), no significant differences were seen for moderate and severe grades of the disease. Likewise, empirical scaling methods (Johnson et al., 2010; Simcyp (Certara) meta-analyses) generated scalars comparable to MPPGL-based scalars in this study in mild to severe stages of cirrhosis. Global sensitivity analysis of the contribution of MPPGL to
variability in exposure to CYP3A substrates with varying hepatic extraction indicated that this parameter has a significant impact, second only to enzyme abundance (used as part of the relative expression factor, REF (Neuhoff et al., 2021), in in vitro to in vivo extrapolation exercises), whereas liver size was the least significant scalar. It is worth noting that, while the impact of changes in liver size on systemic drug exposure was not very significant, variations in liver volume associated with disease progression could affect the extent of hepatic drug exposure, particularly for drugs with localized effects in the liver, such as statins and metformin, where interactions with hepatic transporters are integral to the drug's disposition within the liver.

Our understanding of the impact of NAFLD, across its stages, on drug metabolism, oral bioavailability, systemic exposure, and therapeutic response remains limited. In a clinical investigation involving individuals with biopsy-proven NAFLD, midazolam plasma concentrations were notably higher than those in healthy controls, indicating a 2.4-fold increase in NASH and suggesting a reduction in CYP3A activity (Woolsey et al., 2015). In vitro investigations using liver tissue from individuals with NAFL, NASH, and cirrhosis demonstrated a progressive decline in both the abundance and activity of drug-metabolizing enzymes with the advancement of liver disease (Fisher et al., 2009; Jamwal et al., 2018; El-Khateeb et al., 2021a). These observations combined with the findings of the current study suggest that NAFLD-driven reduction in the drug metabolic capacity of the liver is likely due to changes in the expression and activity in relevant pathways, while the reduced MPPGL is compensated by the reported increase in liver size (Bian et al., 2015; Ozaki et al., 2016). This might not be the case with advanced stages of cirrhosis, where all three parameters are reported to be affected (El-Khateeb et al., 2020, 2021a). These findings underscore the importance of considering the stage of hepatic impairment in individuals with liver disease when attempting to predict alterations in drug metabolism using PBPK modelling.

While the significance of the reported data in modeling drug exposure in NAFLD populations is emphasized, the reliance on opportunistic samples can introduce limitations to the generalizability of
the findings, particularly in relation to the limited sample size in each group and the diverse demographic characteristics of the donors. For example, two out of six NASH patients in the liver biopsy group exhibited a normal body mass index (BMI), indicating the presence of lean NASH in this group, while the mean BMI for donors of the post-mortem NASH liver samples (n = 7) was in the overweight bracket. In addition, variations in cirrhosis from different etiologies are not explored in this report. Finally, information regarding the anatomic location of liver specimens was absent, which may limit the utility of the data, considering the reported heterogeneity of the impact of disease on liver tissue (Arun et al., 2007; Ooi et al., 2021).

In summary, this study reports, for the first time, specific scaling factors in NAFL and NASH patients, revealing significantly lower MPPGL values with disease progression compared with histologically normal livers. Experimentally obtained MPPGL values scaled with liver volume were consistent with empirical methods that account for changes in functional liver volume across the stages of disease progression. The measured values provide additional systems data for PBPK models intended for a priori dosage adjustment in NAFL and NASH patients. This approach aligns with recommendations from various studies (El-Khateeb et al., 2021a; Murphy et al., 2023) advocating evidence-based dose adjustment for special populations in the absence of clinical data (Rostami-Hodjegan and Achour, 2023; El-Khateeb et al., 2021b).
Acknowledgments

The surgical specimens used in this study were provided by the University of Kansas Liver Tissue Biorepository supported by grant 1P20GM144269-01 from the National Institute of General Medical Sciences. The authors acknowledge the contribution of the patients who donated specimens for research as well as the physicians, nurses and researchers who procured the specimens. The post-mortem specimens were provided by Amsbio, LLC, USA, with diagnosis and histopathology information. The authors also thank Dr Matthew Harwood of Certara for his assistance with modelling using the Simcyp® Simulator. Teresa Sierra’s PhD program is funded by the Department of Biomedical and Pharmaceutical Sciences, College of Pharmacy, University of Rhode Island.

Data Availability Statement

All data supporting the findings are included in the manuscript and supplemental file.

Authorship Contributions

Participated in research design: Achour.

Conducted experiments: Sierra.

Performed data analysis: Sierra and Achour.

Wrote or contributed to the writing of the manuscript: Sierra and Achour.
References


Footnotes

This work received no external funding. The authors have no actual or perceived conflict of interest in relation to the contents of this article.
Figure Legends

**Figure 1.** Protein content per gram liver. Protein content in homogenates, S9 fractions and cytosols for normal control compared with NAFL, NASH and NASH with cirrhosis (a). Recovery (b) and enrichment (c) of reticular membrane proteins in liver S9 and microsomal fractions from donors with liver disease. Recovery values measured in microsomes are used to correct for loss of microsomal protein (corrected microsomal protein per gram liver) (d). Data were collated from the post-mortem and surgical sets for each disease group. Data are presented as mean (bars and horizontal lines) and standard deviation (error bars), and statistics used were ANOVA (top annotation), followed by post-hoc pairwise t-tests relative to the reference cohort (bottom annotation): NS, non-significant; *, p < 0.05; **, p < 0.01; ***, p < 0.001. HomPPGL, homogenate protein per gram liver; S9PPGL, S9 protein per gram liver; CPPGL, cytosol protein per gram liver; MPPGL, microsomal protein per gram liver. Sample size in each cohort: n = 36 (normal), n = 27 (NAFL), n = 13 (NASH), and n = 26 (NASH with cirrhosis).

**Figure 2.** Protein content per gram kidney. Protein content in homogenates, S9 fractions and cytosols for normal controls compared with NAFL and NASH samples. Data are presented as mean (horizontal lines) and standard deviation (error bars), and statistics used were ANOVA, followed by post-hoc pairwise t-tests relative to the reference cohort: NS, non-significant. HomPPGK, homogenate protein per gram kidney; S9PPGK, S9 protein per gram kidney; CPPGK, cytosol protein per gram kidney; MPPGK, microsomal protein per gram kidney. Sample size in each cohort: n = 17 (normal), n = 7 (NAFL), and n = 7 (NASH).

**Figure 3.** Covariates of liver (normal) and kidney (all samples) scalars. Analysis of changes in protein content in homogenate, S9, cytosol and microsomal fractions in relation to sex (a), age (b), and BMI (c, d). No sex (t-test, a), age (ANOVA, b) or BMI (ANOVA, d) related differences were observed in protein content in homogenate, S9, cytosol, or microsomal fractions from human liver (normal, n = 36) or kidney (all cohorts, n = 30) tissues. In panel a, the whiskers reflect the ranges, the boxes reflect the 25th and 75th percentiles, the lines are the medians, and the + signs are the means. In panel c, no
differences in BMI range or mean between normal, NAFL, NASH, NASH/cirrhosis groups. F, female; M, male; BMI, body mass index; HLM, human liver microsomes; HKM, human kidney microsomes; NAFL, non-alcoholic fatty liver; NASH, non-alcoholic steatohepatitis. Corrected liver microsomal protein content and uncorrected kidney microsomal protein content were used for the assessment. Liver sample size: n = 36 (normal). Sample size of kidney cohorts: n = 17 (normal), n = 7 (NAFL), and n = 7 (NASH).

**Figure 4.** Covariates of liver scalars in NAFL, NASH and NASH/cirrhosis samples. Analysis of changes in protein content in homogenate, S9, cytosol and microsomal fractions in relation to sex (a), age (b), and BMI (c). No sex (t-test, a), age (ANOVA, b) or BMI (ANOVA, d) related differences were observed in protein content in homogenate, S9, cytosol, or microsomal fractions from human liver tissues across progressive stages of non-alcoholic liver disease. In panel a, the whiskers reflect the ranges, the boxes reflect the 25th and 75th percentiles, the lines are the medians, and the + signs are the means. Abbreviations: F, female; M, male; BMI, body mass index; HLM, human liver microsomes; NAFL, non-alcoholic fatty liver; NASH, non-alcoholic steatohepatitis; cirrhosis represents NASH with cirrhosis. Corrected liver microsomal protein content was used for the assessment. Sample size in each cohort: n = 27 (NAFL), n = 13 (NASH), and n = 26 (NASH with cirrhosis).

**Figure 5.** Summary of global sensitivity analysis (GSA) of the impact of scalars (CYP3A4 abundance, MPPGL and liver size) on variability in exposure (AUC) to oral alprazolam (low hepatic extraction ratio), midazolam (intermediate hepatic extraction ratio), and ibrutinib (high extraction ratio) within minimal PBPK models of the drugs. The relative effect of each factor on exposure variability is expressed as a percentage with the dominant factor being hepatic CYP3A4 abundance, followed by MPPGL. HSA, human serum albumin; MPPGL, microsomal protein per gram liver.
Table 1. Summary of donor demographics and clinical diagnosis. Samples were either surgical (n = 71 livers) or post-mortem (n = 31 matched livers and kidneys).

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Source</th>
<th>Characteristics</th>
<th>Normal</th>
<th>NAFL</th>
<th>NASH</th>
<th>NASH/Cirrhosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>Surgical</td>
<td></td>
<td>19</td>
<td>20</td>
<td>6</td>
<td>26</td>
</tr>
<tr>
<td></td>
<td>Sex</td>
<td>F (11), M (8)</td>
<td></td>
<td></td>
<td></td>
<td>F (13), M (13)</td>
</tr>
<tr>
<td></td>
<td>Age (years)</td>
<td>47.7 ± 19.8</td>
<td>50.2 ± 12.7</td>
<td>50.0 ± 18.9</td>
<td>59.9 ± 9.4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ethnicity</td>
<td>White (17), Black (1), - (1)</td>
<td>White (16), Black (1), - (3)</td>
<td>White (6)</td>
<td>White (23), Black (1), Native American (1), - (1)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>BMI (kg/m²)</td>
<td>33.5 ± 9.9</td>
<td>32.4 ± 8.2</td>
<td>32.2 ± 8.8</td>
<td>31.3 ± 4.7</td>
<td></td>
</tr>
<tr>
<td>Liver</td>
<td>Post-mortem</td>
<td></td>
<td>7</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Sex</td>
<td>F (2), M (15)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Age (years)</td>
<td>60.4 ± 19.1</td>
<td>55.3 ± 14.4</td>
<td>51.9 ± 14.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ethnicity</td>
<td>White (17)</td>
<td>White (7)</td>
<td>White (7)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>BMI (kg/m²)</td>
<td>27.1 ± 4.5</td>
<td>32.4 ± 7.3</td>
<td>28.2 ± 2.2</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Expressed as mean ± standard deviation; matched liver and kidney from the same donors. In parentheses are the numbers of samples in each category. -, data not available. Abbreviations: BMI, body mass index; F, female; M, Male; NAFL, non-alcoholic fatty liver; NASH, non-alcoholic steatohepatitis.*
Table 2. Summary of hepatic scaling factors for NAFL, NASH and NASH with cirrhosis.

<table>
<thead>
<tr>
<th>Tissue source</th>
<th>Cohort</th>
<th>Protein content</th>
<th>Recovery of microsomal protein</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>HomPPGL (mg/g liver)</td>
<td>S9PPGL (mg/g liver)</td>
</tr>
<tr>
<td>Post-mortem</td>
<td>Normal</td>
<td>143.3 ± 19.7 (114.8–191.7)</td>
<td>87.7 ± 15.5 (50.4–109.9)</td>
</tr>
<tr>
<td></td>
<td>NAFL</td>
<td>143.8 ± 13.8 (127.6–168.9)</td>
<td>71.2 ± 11.8 (51.6–85.9)</td>
</tr>
<tr>
<td></td>
<td>NASH</td>
<td>140.6 ± 24.4 (96.4–165.9)</td>
<td>84.8 ± 13.8 (68.2–102.9)</td>
</tr>
<tr>
<td></td>
<td>NASH/Cirrhosis</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Surgical</td>
<td>Normal</td>
<td>130.9 ± 25.4 (69.2–165.3)</td>
<td>102.3 ± 21.2 (51.8–124.7)</td>
</tr>
<tr>
<td></td>
<td>NAFL</td>
<td>137.8 ± 21.6 (68.6–170.1)</td>
<td>107.7 ± 22.1 (42.9–142.8)</td>
</tr>
<tr>
<td></td>
<td>NASH</td>
<td>143.5 ± 12.6 (121.1–156.4)</td>
<td>106.1 ± 26.0 (63.9–134.9)</td>
</tr>
<tr>
<td></td>
<td>NASH/Cirrhosis</td>
<td>115.0 ± 16.0 (77.5–153.3)</td>
<td>84.9 ± 17.4 (51.2–120.3)</td>
</tr>
</tbody>
</table>

Data expressed as mean ± standard deviation and range in parentheses; a only two samples from the post-mortem NAFL group (n = 7) returned good quality activity data for recovery and enrichment. Abbreviations: NAFL, non-alcoholic fatty liver; NASH, non-Alcoholic steatohepatitis; HomPPGL, homogenate protein per gram liver; CPPGL, cytosolic protein per gram liver; S9PPGL, S9 protein per gram liver; MPPGL, microsomal protein per gram liver.
Table 3. Summary of kidney scaling factors for NAFL, NASH and NASH with cirrhosis.

<table>
<thead>
<tr>
<th>Tissue source</th>
<th>Cohort</th>
<th>HomPPGK (mg/g kidney)</th>
<th>S9PPGK (mg/g kidney)</th>
<th>CPPGK (mg/g kidney)</th>
<th>MPPGK (mg/g kidney)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Normal</td>
<td>100.9 ± 16.2 (72.5–124.7)</td>
<td>60.0 ± 14.4 (35.1–81.7)</td>
<td>47.6 ± 11.2 (29.4–63.2)</td>
<td>10.4 ± 2.3 (6.6–15.0)</td>
</tr>
<tr>
<td></td>
<td>NAFL</td>
<td>94.7 ± 18.9 (73.4–125.6)</td>
<td>53.0 ± 18.0 (32.6–77.9)</td>
<td>44.7 ± 11.8 (26.8–58.2)</td>
<td>11.3 ± 3.3 (7.9–17.0)</td>
</tr>
<tr>
<td></td>
<td>NASH</td>
<td>102.0 ± 19.2 (62.4–121.8)</td>
<td>67.2 ± 12.8 (56.4–84.9)</td>
<td>50.2 ± 11.9 (39.4–68.4)</td>
<td>12.1 ± 1.3 (10.5–13.3)</td>
</tr>
<tr>
<td></td>
<td>NASH/Cirrhosis</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

Data expressed as mean ± standard deviation and range in parenthesis. Abbreviations: NAFL, non-alcoholic fatty liver; NASH, non-alcoholic steatohepatitis. HomPPGK, homogenate protein per gram kidney; CPPGK, cytosolic protein per gram kidney; S9PPGK, S9 protein per gram kidney; MPPGK, microsomal protein per gram kidney.
Table 4. MPPGL-based hepatic scaling factors for NAFL, NASH and cirrhosis relative to healthy control from the current study and previous work compared with empirical scaling.

<table>
<thead>
<tr>
<th>Cohort</th>
<th>Scalar from the Current Study: ((\text{MPPGL}<em>{\text{Cohort}} \times \text{Total Liver Volume}</em>{\text{Population}})^{a}) Relative to Healthy Cohort</th>
<th>Scalar from (El-Khateeb et al., 2020): ((\text{MPPGL}<em>{\text{Cohort}} \times \text{Total Liver Volume}</em>{\text{Population}})^{b}) Relative to Healthy Cohort</th>
<th>Empirical Scalar: ((\text{MPPGL}<em>{\text{Healthy}} \times \text{Functional Liver Volume}</em>{\text{Population}})^{c}) Relative to Healthy Population</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>NAFL</td>
<td>0.96</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>NASH</td>
<td>0.99</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Cirrhosis A</td>
<td>0.84</td>
<td>0.74</td>
<td>0.81; 0.89; 0.86</td>
</tr>
<tr>
<td>Cirrhosis B</td>
<td>0.61</td>
<td>0.77</td>
<td>0.65; 0.71; 0.71</td>
</tr>
<tr>
<td>Cirrhosis C</td>
<td>0.44</td>
<td>0.57</td>
<td>0.53; 0.61; 0.59</td>
</tr>
<tr>
<td>Cirrhosis A–C</td>
<td>0.63</td>
<td>0.69</td>
<td>0.66; 0.74; 0.72</td>
</tr>
</tbody>
</table>

Data are expressed as a ratio relative to healthy control. \(^{a}\) Changes in total liver volume in NAFLD and cirrhosis from Bian et al., 2015 and Ozaki et al., 2016; \(^{b}\) Changes in total liver volume in cirrhosis from Ozaki et al., 2016; \(^{c}\) Values based on meta-analyses of published functional liver volume data from imaging techniques by Simcyp (Certara), UK; \(^{d}\) Cirrhosis A–C data calculated as a mean based on values for each disease class. Changes in liver volume: 128% (NAFL), 128% (NASH), 102% (Cirrhosis-A), 92% (Cirrhosis-B) and 69% (Cirrhosis-C) of mean healthy liver volume. Abbreviations: NAFL, non-alcoholic fatty liver; NASH, non-alcoholic steatohepatitis; MPPGL, microsomal protein per gram liver.
Fig 1
Fig 2
Fig 3
Fig 4