Considerations for Physiologically Based Modeling in Liver Disease: From Nonalcoholic Fatty Liver (NAFL) to Nonalcoholic Steatohepatitis (NASH)

William A. Murphy[1], Jeffry Adiwidjaja[1,2], Noora Sjöstedt[3], Kyunghee Yang[4], James J. Beaudoin[4], Jessica Spires[2], Scott Q. Siler[4,*], Sibylle Neuhoff[5,*] and Kim L.R. Brouwer[1,*]

Nonalcoholic fatty liver disease (NAFLD), representing a clinical spectrum ranging from nonalcoholic fatty liver (NAFL) to nonalcoholic steatohepatitis (NASH), is rapidly evolving into a global pandemic. Patients with NAFLD are burdened with high rates of metabolic syndrome-related comorbidities resulting in polypharmacy. Therefore, it is crucial to gain a better understanding of NAFLD-mediated changes in drug disposition and efficacy/toxicity. Despite extensive clinical pharmacokinetic data in cirrhosis, current knowledge concerning pharmacokinetic alterations in NAFLD, particularly at different stages of disease progression, is relatively limited. *In vitro*-to-*in vivo* extrapolation coupled with physiologically based pharmacokinetic and pharmacodynamic (IVIVE-PBPK/PD) modeling offers a promising approach for optimizing pharmacologic predictions while refining and reducing clinical studies in this population. Use of IVIVE-PBPK to predict intra-organ drug concentrations at pharmacologically relevant sites of action is particularly advantageous when it can be linked to pharmacodynamic effects. Quantitative systems pharmacology/toxicology (QSP/QST) modeling can be used to translate pharmacokinetic and pharmacodynamic data from PBPK/PD models into clinically relevant predictions of drug response and toxicity. In this review, a detailed summary of NAFLD-mediated alterations in human physiology relevant to drug absorption, distribution, metabolism, and excretion (ADME) is provided. The application of literature-derived physiologic parameters and ADME-associated protein abundance data to inform virtual NAFLD population development and facilitate PBPK/PD, QSP, and QST predictions is discussed along with current limitations of these methodologies and knowledge gaps. The proposed methodologic framework offers great potential for meaningful prediction of pharmacological outcomes in patients with NAFLD and can inform both drug development and clinical practice for this population.

The human liver is a vital organ involved in the synthesis and metabolism of biological molecules, including proteins, and regulation of critical physiologic functions. From a pharmacological perspective, the liver is the primary site for metabolism and clearance of many therapeutics. Hepatocytes, which are the predominant cell type in the liver, are densely populated with membrane transporters and metabolic enzymes to facilitate drug uptake, metabolism, and excretion. Whereas the liver is a highly resilient and regenerative organ, sustained damage to the hepatic parenchyma due to disease or injury can have significant implications for pharmacokinetics. Chronic liver disease (CLD) is estimated to impact 1.5 billion people worldwide, and accounts for 2 million deaths each year.[1] Cirrhosis, the most prevalent severe complication of CLD, is generally characterized by irreversible hepatic scarring, altered hepatic parenchymal and vascular architecture, and impaired liver function. There are several known etiologies of cirrhosis, with viral hepatitis, alcoholic liver disease, and nonalcoholic fatty liver disease (NAFLD) being the most common. Although global epidemiological data on cirrhosis are rather limited, the estimated prevalence in North America ranges from 300 to 1,000 per 100,000 people with relatively higher rates observed among those of African and Hispanic ancestry, and those with less education. Interestingly, CLD mortality over the past decade has increased disproportionately in younger patients, women, and those of European or Native American descent.[1] Despite significant advances in hepatitis vaccination and treatment over the past two decades, several high-income countries have reported increased rates of CLD and cirrhosis alongside higher prevalence of the metabolic syndrome (e.g., obesity and type 2 diabetes mellitus [T2DM]).[1]

Nonalcoholic fatty liver disease, which is strongly associated with the metabolic syndrome, is a leading cause of CLD and

[1]Division of Pharmacotherapy and Experimental Therapeutics, UNC Eshelman School of Pharmacy, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina, USA; [2]Simulations Plus, Inc., Lancaster, California, USA; [3]Division of Pharmaceutical Biosciences, Faculty of Pharmacy, University of Helsinki, Helsinki, Finland; [4]DILIsym Services Division, Simulations Plus Inc., Research Triangle Park, North Carolina, USA; [5]Certara UK Ltd. (Simcyp Division), Sheffield, UK. *Correspondence: Kim L.R. Brouwer (kbrouwer@unc.edu), Sibylle Neuhoff (sibylle.neuhoff@certara.com) and Scott Q. Siler (scott.siler@simulations-plus.com)

Received December 17, 2021; accepted April 5, 2022. doi:10.1002/cpt.2614

cirrhosis globally.[1,2] NAFLD is a progressive spectrum of disease encompassing nonalcoholic fatty liver (NAFL; i.e., simple steatosis), and nonalcoholic steatohepatitis (NASH). NAFLD is diagnosed when steatosis, or fat accumulation, is present in more than 5% of hepatocytes in the absence of excessive alcohol consumption or other causes of steatosis.[3] Diagnosis of NASH, the advanced form of NAFLD, relies on biopsy-based histological observation of steatosis, hepatocellular ballooning, and inflammation.[3] Hepatic fibrosis is associated with NAFLD progression and comorbidity and mortality.[2] NASH with fibrosis can further progress to cirrhosis and/or hepatocellular carcinoma; liver transplantation may be required in some cases.[3] Worldwide prevalence of NAFLD is now estimated to be 25% of the adult population, with 3–5% estimated to have NASH.[2] There is, however, considerable variability in these estimates worldwide. For example, NAFLD prevalence is estimated to be as low as 13.5% in Africa and as high as 33.9% in Asia. In the United States, NAFLD is estimated to impact up to 30% of the population, with the highest rates seen in those of Hispanic ancestry and the lowest rates observed in those of African ancestry.[3] Similar demographic/racial trends have been observed with the metabolic syndrome in the United States, which currently impacts ~ 30% of the population and alarmingly, 70.7% of those with NASH globally.[2] Fueled by a global epidemic of the metabolic syndrome, NAFLD and NASH prevalence are projected to increase by 21% and 63%, respectively, by 2030 and become the leading cause of liver transplantation in the United States by 2025.[1] Notably, there are currently no regulatory-approved pharmacologic treatments for NAFLD. Nevertheless, patients with NAFLD often take multiple medications to treat comorbid disease states related to the metabolic syndrome[4] (hyperlipidemia (69% of NAFLD patients), obesity (51%), hypertension (39%), and T2DM (23%)[2]). Whereas the impact of cirrhosis on clinical pharmacokinetics is well-established, with the first documented study occurring over 60 years ago,[5] current knowledge regarding the impact of NAFLD on pharmacokinetics is comparatively very limited and often overlooked in clinical practice and drug development. With projections of an exponential increase in NAFLD cases over the next decade, a better understanding of the impact of this disease on drug disposition and clinical drug response is needed. Moreover, the increasing prevalence of NAFLD in pediatric patients[6] warrants further characterization of pharmacokinetic changes in this subpopulation.

Hepatic impairment is associated with a significant decline in metabolic clearance capacity and intra- and extrahepatic physiologic changes.[7] Many marketed drugs undergo significant hepatic clearance. However, clinical dosing is typically optimized in "healthy" populations.[7] Although regulatory agencies strongly encourage the study of hepatic impairment in premarketing clinical trials, many hepatically cleared drugs approved by the US Food and Drug Administration (FDA) over the past two decades lack explicit dosing recommendations for this population.[7] Additionally, regulatory labeling and guidance documents pertaining to pharmacokinetic alterations in liver disease are based primarily on Child-Pugh score classification.[8,9] Current dosing recommendations can be agnostic to CLD etiology,[7] which may differentially impact pharmacokinetics.[10,11] While NAFLD is a common cause of cirrhosis, only about 20% of patients with NASH progress to cirrhosis in their lifetime.[3] Despite increasing NAFLD prevalence in modern society and consistent data demonstrating progressive NAFLD-mediated alterations in drug metabolizing enzymes (DMEs) and transporters,[12] patients with non-cirrhotic fatty liver disease often are overlooked in premarketing pharmacokinetic studies and, accordingly, lack regulatory-approved labeling recommendations for dosing. A manual search of FDA-approved drug labels using the Drugs@FDA database (https://www.accessdata.fda.gov/scripts/cder/daf/) revealed that 144 of 233 (62%) small molecule new molecular entities approved from January 1, 2015, to November 23, 2021, had dosing recommendations for general hepatic impairment or specific Child-Pugh classification(s), or had reported pharmacokinetic data in the hepatically impaired population. During the same timeframe, zero small molecule drugs receiving first time FDA-approval had dosing recommendations or reported pharmacokinetic data for patients with non-cirrhotic NAFLD. Premarketing clinical trials for drugs not indicated for the treatment of liver disease typically exclude patients with elevated liver enzymes, posing a barrier for adequate study of the NAFLD population during drug development. Although not all patients with NAFLD present with elevated liver enzymes, regardless of histological staging,[3] clinical trials rarely screen participants for NAFLD due to the highly invasive nature of a liver biopsy, which is the "gold standard" for proper diagnosis. As a result, undiagnosed/asymptomatic patients with NAFLD have the potential to be included in premarketing clinical trials without being properly identified. Therefore, the effect of NAFLD on a new molecular entity's pharmacokinetic profile is often unknown when it receives regulatory approval. Although the FDA has published draft guidance documents on pharmacokinetic considerations for drugs designed specifically to treat NASH in both patients with cirrhotic[13] and non-cirrhotic[14] NASH during clinical development, there is currently no guidance for the evaluation of drugs used to treat comorbid diseases in the NAFLD population (e.g., antidiabetics, antihypertensives, and antihyperlipidemics). Therefore, there is a scarcity of clinical data, particularly for medications indicated to treat other disease states commonly observed in the NAFLD population, despite known changes in absorption, distribution, metabolism, and excretion (ADME; **Figure 1**).

Physiologically based pharmacokinetic/pharmacodynamic (PBPK/PD) modeling is a computational methodology used to predict pharmacokinetic and pharmacodynamic profiles for different scenarios (e.g., dose optimization, drug–drug interactions [DDIs], and target-occupancy), with the primary goal of refining, reducing, and, if possible, replacing the need for time-consuming and costly clinical studies specifically in vulnerable patient populations. Physiologically scaled *in vitro* parameters can be utilized effectively to inform clinical predictions (e.g., via *in vitro*-to-*in vivo* extrapolation coupled with PBPK/PD [IVIVE-PBPK/PD] modeling)[15]; this approach is now used frequently to facilitate regulatory decision making and expedite clinical development.[16] Although relatively few FDA submissions to date have used PBPK data to inform dosing in hepatically impaired populations, it has been proposed as a valuable tool to better understand pharmacokinetic changes associated with generalized hepatic impairment and cirrhosis.[7] A key feature of IVIVE-PBPK is its ability to incorporate

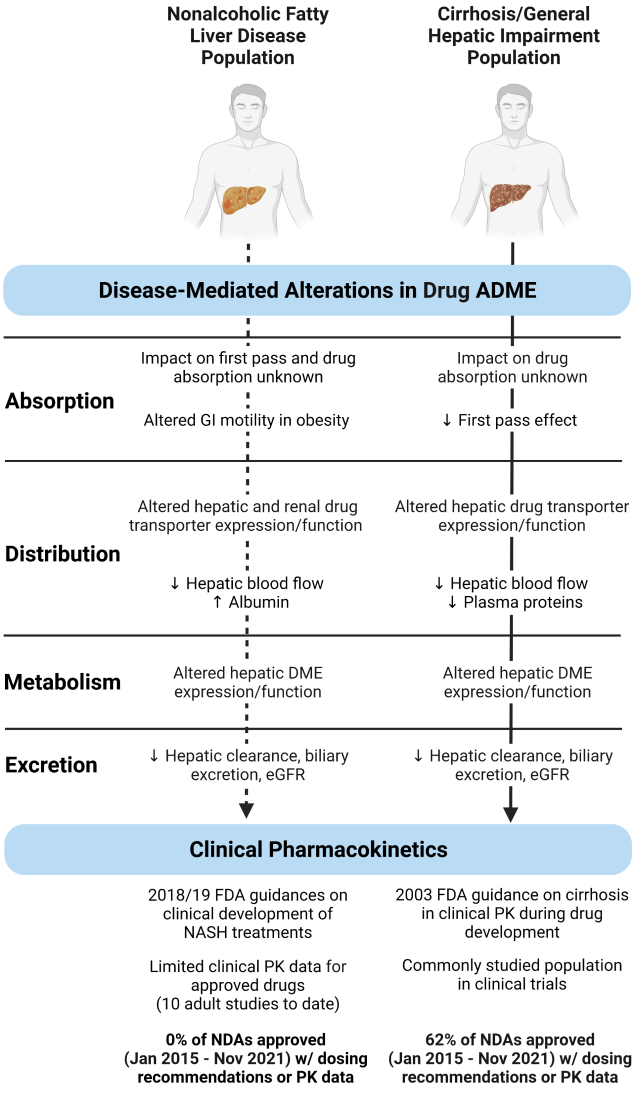

Figure 1 Disease-mediated alterations in drug ADME parameters. Documented changes in ADME-related physiologic characteristics have been reported in the NAFLD and cirrhosis populations. How these changes translate to differences in clinical pharmacokinetics relative to a healthy liver population is largely unknown in NAFLD, despite extensive efforts over the past few decades to characterize pharmacokinetic alterations in cirrhosis.
Created with BioRender.com. ADME, absorption, distribution, metabolism, excretion; DME, drug metabolizing enzyme; eGFR, estimated glomerular filtration rate; FDA, Food and Drug Administration; GI, gastrointestinal; NAFL, nonalcoholic fatty liver; NAFLD, nonalcoholic fatty liver disease (encompasses NAFL and NASH); NASH, nonalcoholic steatohepatitis; PK, pharmacokinetics.

known physiologic and physiochemical drug parameters to predict pharmacokinetics in a specific population. While PBPK models have been developed retrospectively in select cases to accurately capture pharmacokinetic profiles in NAFLD,[17,18] there is an increasing need to evaluate DDIs and other real-world scenarios in this population. By leveraging documented physiologic changes associated with NAFLD and its progression, IVIVE-PBPK/PD modeling has the potential to make informative predictions in this highly heterogenous and polypharmacy population while limiting substantial investment in *de novo* clinical studies.

A literature review focused on clinical studies in NAFLD published up until November 23, 2021, was performed using the PubMed database (https://pubmed.ncbi.nlm.nih.gov/), the World Wide Web, and Google Scholar with the use of generic search terms in various combinations pertaining to the topic of interest (e.g., "nonalcoholic fatty liver disease," "hepatic impairment," "physiologically based pharmacokinetic modeling," "quantitative systems pharmacology," "drug metabolizing enzyme," and "drug transporter"). Only studies with both control and NAFLD groups, or those matched to a study population with similar demographics/ethnic makeup, were collated in **Tables 1-4** and **Supplementary Table S1**. Studies without an appropriate control or NAFLD group, or separate study with similar demographics/ethnic makeup, were confined to **Supplemental Data 1 and 2**. This review highlights our current understanding of physiologic changes that are relevant to pharmacokinetics and PBPK/PD modeling of small molecule drugs over the progressive course of NAFLD to facilitate both drug development for novel therapeutics in this population and clinical treatment of metabolic syndrome-related comorbidities. Quantitative systems pharmacology/toxicology (QSP/QST) methods to supplement PBPK/PD modeling are discussed as a tool to translate predicted drug concentrations and effects into pharmacological outcomes.

PHYSIOLOGIC CHANGES ASSOCIATED WITH NAFLD

Microphysiologic hepatic changes associated with NAFLD

Dietary, environmental, and genetic factors are hypothesized to initiate NAFLD development via the expansion of dysfunctional adipose tissue, alterations in the intestinal microbiome, increases in circulating cholesterol and free fatty acids (FFAs), and development of insulin resistance.[19] Insulin resistance results in excessive levels of insulin and glucose, exacerbation of adipocyte dysfunction, stimulation of lipolysis and alterations in the release of adipokines (e.g., leptin elevations and adiponectin reductions), and pro-inflammatory cytokines including interleukin 6 (IL-6) and tumor necrosis factor-α that can maintain a state of insulin resistance.[19] Elevated serum FFAs and hepatic insulin resistance-mediated increases in *de novo* lipogenesis, along with other alterations in hepatic lipid disposition (e.g., decreased β-oxidation of FFAs, decreased very low density lipoprotein-triglyceride assembly, and increased hepatic triglyceride accumulation) lead to increased hepatic FFAs.[19] Increases in saturated FFAs generate oxidative stress, which, in turn, affects hepatic cell function and viability, thereby activating immune cells (e.g., macrophages and neutrophils), inflammatory pathways, and the development of fibrosis via hepatic stellate cell-mediated collagen deposition.[20] These processes are believed to play a central role in continued promotion of hepatic and systemic abnormalities in NAFLD, which can develop into extrahepatic disorders.[20]

NAFLD grades/stages are classified using the semiquantitative NAFLD activity score (NAS)[6] and noninvasive fibrosis scoring systems[3] (i.e., NAFLD fibrosis score and fibrosis-4 index). NAS (0–8) is the sum total of numerical scores applied to three key histological features: steatosis (0–3), lobular inflammation (0–3), and hepatocellular ballooning (0–2). Steatosis (i.e., infiltration of liver cells with fat) visible in > 5% of hepatocytes by light microscopy is

Table 1 Summary of physiologic changes in NAFLD

Parameter (units)	Study	Control group (sample size)	Disease group (sample size)	Mean fold-change relative to control
Adult population				
Liver size, composition, and scaling factors				
Liver weight (kg)[a]	Jamwal *et al*. (2018)[30]	"Normal" (non-steatotic) liver (*n* = 24)	NAFL (*n* = 26)	1.3
			NASH, cirrhosis status NR (*n* = 24)	1.2
Liver fat (%)	Lambert *et al*. (2014)[85]	Metabolic syndrome w/o NAFLD (*n* = 11)	Metabolic syndrome w/ NAFLD (*n* = 13)	5.9[b]
	Maximos *et al*. (2015)[86]	Obese non-NAFLD (*n* = 60)	NAFLD (normal ALT), cirrhosis status NR (*n* = 165)	6.2[b,c]
			NAFLD (elevated ALT), cirrhosis status NR (*n* = 215)	9.2[b,c]
CPPGL (mg cytosolic protein/g liver)	El-Khateeb *et al*. (2020)[31]	Noncirrhotic cohort w/ liver metastasis (*n* = 13)	NASH w/cirrhosis (*n* = 8)	0.9
MPPGL (mg microsomal protein/g liver)	El-Khateeb *et al*. (2020)[31]	Noncirrhotic cohort w/ liver metastasis (*n* = 13)	NASH w/cirrhosis (*n* = 8)	0.7[b]
S9PPGL (mg S9 protein/g liver)	El-Khateeb *et al*. (2020)[31]	Noncirrhotic cohort w/ liver metastasis (*n* = 13)	NASH w/cirrhosis (*n* = 8)	0.8
Cardiac output and liver blood flow				
Cardiac output (L/min)	Houghton *et al*. (2019)[24]	Healthy subjects (*n* = 34)	NAFLD, cirrhosis status NR (*n* = 46)	1.0
	VanWagner *et al*. (2020)[23]	Healthy subjects (*n* = 1,668)	NAFLD, cirrhosis status NR (*n* = 159)	1.2[b]
Hepatic arterial blood flow (mL/min/100 mL of liver tissue)	Shigefuku *et al*. (2012)[25]	NR	NAFL (*n* = 9)	0.7[d]
			Early-stage NASH w/o bridging fibrosis (*n* = 38)	0.5[d]
			Late-stage NASH w/ bridging fibrosis, ± cirrhosis (*n* = 18)	0.5[d]
Portal vein blood flow (mL/min/100 mL of liver tissue)	Shigefuku *et al*. (2012)[25]	NR	NAFL (*n* = 9)	0.6[d]
			Early-stage NASH w/o bridging fibrosis (*n* = 38)	0.5[d]
			Late-stage NASH w/ bridging fibrosis, ± cirrhosis (*n* = 18)	0.4[d]
Total hepatic blood flow (mL/min/100 mL of liver tissue)	Shigefuku *et al*. (2012)[25]	NR	NAFL (*n* = 9)	0.7[d]
			Early-stage NASH w/o bridging fibrosis (*n* = 38)	0.5[d]
			Late-stage NASH w/ bridging fibrosis, ± cirrhosis (*n* = 18)	0.5[d]
Kidney function				
Estimated glomerular filtration rate (mL/min/1.73 m^2)	Targher *et al*. (2010)[32]	Healthy subjects (*n* = 80)	NASH w/o cirrhosis (*n* = 80)	0.9[b]
	Kasumov *et al*. (2011)[87]	Healthy subjects (*n* = 25)	NAFL (*n* = 11)	0.9
			NASH, cirrhosis status NR (*n* = 24)	1.0
	Choudhary *et al*. (2016)[88]	Healthy subjects (*n* = 186)	NAFL (*n* = 187)	1.0

(Continued)

Table 1 (Continued)

Parameter (units)	Study	Control group (sample size)	Disease group (sample size)	Mean fold-change relative to control
Plasma proteins and hematocrit				
α1-Acid glycoprotein (g/L)	Koruk *et al*. (2003)[26]	Healthy subjects (*n* = 16)	NASH w/o cirrhosis (*n* = 18)	1.0
	Priya *et al*. (2010)[27]	Non-CAD control group (*n* = 28)	NASH w/o cirrhosis (*n* = 18)	1.0
Serum albumin (g/dL)	Koruk *et al*. (2003)[26]	Healthy subjects (*n* = 16)	NASH w/o cirrhosis (*n* = 18)	1.1
	Priya *et al*. (2010)[27]	Non-CAD control group (*n* = 28)	NASH w/o cirrhosis (*n* = 18)	1.1
Hematocrit (%)	Das *et al*. (2011)[89]	Healthy subjects (*n* = 77)	NAFLD, cirrhosis status NR (*n* = 105)	1.1[b]
	Li *et al*. (2012)[90]	Healthy subjects (*n* = 1,231)	NAFLD, cirrhosis status NR (*n* = 590)	1.1[b]
	Issa *et al*. (2014)[91]	Healthy subjects (*n* = 18)	NAFL (*n* = 30)	1.1
			NASH, cirrhosis status NR (*n* = 47)	1.0
	Li *et al*. (2014)[28]	Healthy subjects (*n* = 110)	NAFL (*n* = 108)	1.1[b]
			NASH ± cirrhosis (*n* = 107)	1.2[b]
Pediatric population				
Liver fat (%)	Di Costanzo *et al*. (2019)[92]	Non-NAFLD obese (*n* = 125)	NAFLD, cirrhosis status NR (*n* = 105)	10.0[b]
Cardiac output (mL/min/m^2)	Manco *et al*. (2009)[93]	Healthy subjects (*n* = 11)	NAFLD, cirrhosis status NR (*n* = 20)	1.0
		Non-NAFLD obese (*n* = 20)		0.9
Estimated glomerular filtration rate (mL/min/1.73 m^2)	Pacifico *et al*. (2016)[94]	Healthy subjects (*n* = 130)	NAFLD, cirrhosis status NR (*n* = 268)	1.1[b]
		Non-NAFLD obese (*n* = 328)		1.0
	Di Costanzo *et al*. (2019)[92]	Non-NAFLD obese (*n* = 125)	NAFLD, cirrhosis status NR (*n* = 105)	1.0

See **Supplementary Data 1** for detailed clinical information for control/comparator and disease groups. See **Supplementary Data 2** for reported study values and further information on the fold-change calculations of physiologic parameters and additional physiological data from studies lacking healthy controls or a NAFLD group.

ALT, alanine aminotransferase; CAD, coronary artery disease; CPPGL, cytosolic protein per gram of liver; kg, kilogram; mL, milliliter; MPPGL, microsomal protein per gram of liver; NAFL, nonalcoholic fatty liver (i.e., simple steatosis); NAFLD, nonalcoholic fatty liver disease (encompasses NAFL and NASH); NASH, nonalcoholic steatohepatitis; NR, not reported; S9PPGL, S9 protein per gram of liver; w/, with; w/o, without; ±, with and without.

[a]Calculated from liver volume assuming a liver tissue density of 1.08 kg/L.[95] [b]Statistically significant difference ($P < 0.05$) compared with control group as reported in the referenced study. [c]Data for control group extracted using WebPlotDigitizer version 4.2 (https://apps.automeris.io/wpd/). [d]Comparison to separate study with presumed similar ethnic makeup based on geographical location of study, but differences in age and sex (see **Supplementary Data 2**). No statistics available.

generally used to characterize fatty liver. Lobular inflammation is categorized based on the number of Kupffer cell aggregates with or without fat globules, and clusters of mononuclear cells, neutrophils, and/or eosinophils. Hepatocellular ballooning is a phenomenon related to liver cell injury involving cell enlargement, cytoplasm rounding, rearrangement of the intermediate filament cytoskeleton, and presence of keratin 8/18-positive ubiquitinated filaments known as Mallory–Denk bodies.[6] Patients with a ballooning score > 0 have a reduced number of viable hepatocytes, which can significantly impact drug disposition. Whereas NAS of ≥ 5 correlates with a diagnosis of NASH, NAS < 3 correlates with a "no NASH" diagnosis.[6] Fibrosis (0–4, with cirrhosis corresponding to a score of "4") is assessed separately from NAS, because fibrosis is believed to be less reversible than the aforementioned features, and results from disease activity.[6] Liver stiffness, as measured by magnetic resonance elastography, is used to estimate liver collagen levels associated with the various fibrosis stages.

More recently, likely roles for bile acids (BAs) in NAFLD have emerged.[21] BAs, which are elevated in NASH,[22] are ligands of Takeda G-protein-coupled receptor 5 (TGR5) and the farnesoid X receptor (FXR).[21] Upon activation, the widely expressed TGR5 participates in the regulation of energy expenditure,

glucose metabolism, and immunity.[21] Activation of FXR, which is highly expressed in the liver and intestine, regulates BA homeostasis, along with the up- and downregulation of multiple hepatic drug and BA transporters. Furthermore, activated FXR represses *de novo* lipogenesis and stimulates fatty acid β-oxidation, thereby restricting hepatic lipid accumulation; on the other hand, FXR activation increases hepatic cholesterol due to FXR-mediated downregulation of cytochrome P450 (CYP) 7A1, the rate-limiting enzyme for converting cholesterol to BAs.[21] BA-focused treatment of NAFLD holds much promise, but TGR5/FXR selectivity and toxicity challenges need to be overcome.[21]

Macrophysiologic changes associated with NAFLD

Physiologic changes that affect drug pharmacokinetics must be considered to reliably model drug disposition. Many of the physiologic changes in NAFLD are related to changes mediated by obesity. However, some changes, especially those related to the liver and kidneys, may be specific to NAFLD progression (**Table 1**).

Drugs distribute in the body through the systemic circulation, which can be affected by changes in blood flow. Mean cardiac output has been shown to increase in NAFLD by 17%, but this is primarily explained by obesity[23] and no difference in cardiac output between NAFLD and non-NAFLD sedentary controls was reported in a smaller study.[24] Liver blood flow, which is especially important for first-pass elimination of drugs, is decreased in NAFLD. Portal vein and total hepatic blood flow decrease up to 30% as the degree of fibrosis increases in NASH compared with NAFL.[25] Serum albumin concentrations increase 8–11%, whereas changes in α1-acid glycoprotein concentrations are negligible in NASH (**Table 1**).[26,27] Slight increases in hematocrit (4–18%) have been reported in NAFLD (**Table 1**). Interestingly, hematocrit increases with fibrosis severity in NASH[28] despite known decreases in hematocrit in cirrhosis relative to controls,[29] although this association is likely confounded by the presence of variceal bleeding in the cirrhotic cohort. When modeling disease progression, it is important to consider changes in liver parameters that are relevant for IVIVE of transport and metabolism. One of these IVIVE scalars, the liver weight, is increased by ~ 20% in NAFLD.[30] However, in cirrhosis, the liver weight is decreased and cirrhotic liver samples from subjects with NAFLD showed 0.74 and 0.88 fold-changes in the microsomal and cytosolic protein per gram liver values, respectively, compared with non-NAFLD control livers (36.6 to 27.0 mg microsomal protein/g liver and 75.4 to 66.4 mg cytosolic protein/g liver, respectively).[31] Information on changes in the number of hepatocytes per gram of liver (i.e., hepatocellularity) in NAFLD has not been published.

In addition to physiologic changes in the liver, drug disposition may be impacted by altered intestinal absorption and/or renal elimination in NAFLD. NAFLD has been associated with an increased risk of stage ≥ 3 chronic kidney disease (CKD) compared with non-NAFLD controls.[32] A reduction in the estimated glomerular filtration rate was documented in adult patients with biopsy-verified NASH relative to healthy controls with matching body mass index (BMI; **Table 1**) independent of traditional CKD risk factors,[32] suggesting a true relationship between NAFLD and CKD development. Gastrointestinal physiology may be directly impacted by obesity resulting in altered absorption; additional physiologic changes are induced when patients undergo bariatric surgery for weight loss.[33] This is important because 80% of patients undergoing bariatric surgery have NAFLD.[34] NAFLD is also associated with an increased risk of gastroesophageal reflux disease[35] and proton pump inhibitors are commonly prescribed in this population.[4] Thus, changes in gastrointestinal pH in NAFLD may result in altered ionization and absorption of drugs. Furthermore, altered gastrointestinal motility has been reported in obesity.[33]

NAFLD-MEDIATED ALTERATIONS IN HEPATIC METABOLIC ENZYMES

NAFLD has differential effects on protein levels of CYPs and non-CYP enzymes (**Table 2**). The abundance of CYP3A4, CYP3A5, CYP1A2, CYP2C19, and CYP2C8 in livers from NAFLD patients was considerably lower than in non-NAFLD cohorts, and the extent of reductions in protein levels appeared to correspond with disease severity (NAFL vs. NASH). There was a trend for reduced CYP2D6 protein levels in patients with NAFLD compared with non-NAFLD control groups, albeit to a lesser extent than that of the other affected CYP enzymes. Conversely, CYP2A6 and CYP2E1 abundance seemed to be unaffected by NAFLD. The trend for disease-related changes in CYP2B6 abundance in patients with NAFLD across different studies was less clear than the other CYP enzymes. Protein levels of CYP27A1, which is involved in BA synthesis from cholesterol, were unchanged in the livers of NAFLD patients whereas quantitative data on CYP7A1 is lacking. Protein levels of non-CYP enzymes in patients with NAFLD were relatively unchanged compared with a non-NAFLD cohort, except for several sulfotransferases (SULT1A1 and SULT1C4) and uridine 5'-diphospho-glucuronosyltransferases (UGTs; **Table 2**).

A stratification of hepatic CYP3A4 abundance based on diabetic status hinted at a more prominent contribution of intrinsic NAFLD factors than T2DM to the overall reduction in CYP3A4 protein levels. The mean values of CYP3A4 abundance were lowered by ~ 65% and ~ 40% in NASH patients with and without T2DM, respectively, compared to non-NAFLD individuals with a similar BMI distribution.[30] A correlation between obesity and higher CYP2E1 protein levels and activity compared to lean individuals has been established.[36] Similar abundance of CYP2E1 in patients with NAFLD compared with a BMI-matched non-NAFLD cohort suggests that obesity was solely responsible for apparent changes in CYP2E1 activity in NAFLD patients. Hence, CYP2E1 activity in the lean NAFLD patient population, which represents ~ 25% of individuals with NAFLD and is particularly prevalent in those of East and South Asian ancestry,[37] is likely to be comparable to that of healthy non-obese individuals. Interestingly, elevated hepatic CYP2E1 protein levels in obesity have been linked with induction of reactive oxygen species formation in the presence of liver fat accumulation,[38] suggesting that CYP2E1 may play a role in NAFLD disease progression.

Trends of NAFLD-associated changes in protein levels of CYP and non-CYP enzymes generally were consistent across different studies included in **Table 2**, although some variation

Table 2 NAFLD-mediated alterations in human hepatic metabolic enzyme protein levels

Enzyme	Study	Trend change in protein levels with increasing disease severity (technique, sample type)	Fold-change in protein levels relative to control by disease group		
			NAFL	NASH w/o cirrhosis	NASH w/ cirrhosis
Cytochrome P450 enzymes					
CYP1A2	Fisher *et al*. (2009)[96,a]	↓[b] (WB, HLM)	0.9	0.2	0.4
	Jamwal (2018)[39]	↓ (LFAPQ, HLM)	0.5[c,d]	0.6[c,d]	NR
	El-Khateeb *et al*. (2021a)[48]	↓ (QTAP, HLM)	NR	NR	0.4[e]
CYP2A6	Fisher *et al*. (2009)[96,a]	↑[b] (WB, HLM)	1.2	1.0	2.7
	Jamwal (2018)[39]	↔ (LFAPQ, HLM)	0.7[c]	0.9[c]	NR
	El-Khateeb *et al*. (2021a)[48]	↔ (QTAP, HLM)	NR	NR	1.0[e]
	El-Khateeb *et al*. (2021b)[42]	↓ (QTAP, HLM)	NR	NR	0.3[c,d]
CYP2B6	Fisher *et al*. (2009)[96,a]	↑/↔ (WB, HLM)	2.1	1.8	1.1
	Jamwal (2018)[39]	↓ (LFAPQ, HLM)	0.6[c]	0.7[c]	NR
CYP2C8	Fisher *et al*. (2009)[96,a]	↓[b] (WB, HLM)	1.1	0.7	0.6
	Kakehashi *et al*. (2017)[97]	↓ (RQP, FFPE)	NR	NR	0.6[d]
	Jamwal (2018)[39]	↔ (LFAPQ, HLM)	0.7[c]	0.9[c]	NR
	El-Khateeb *et al*. (2021a)[48]	↓ (QTAP, HLM)	NR	NR	0.7[e]
	El-Khateeb *et al*. (2021b)[42]	↓ (QTAP, HLM)	NR	NR	0.4[c,d]
CYP2C9	Fisher *et al*. (2009)[96,a]	↔ (WB, HLM)	1.4	0.9	1.1
	Kakehashi *et al*. (2017)[97]	↔ (RQP, FFPE)	NR	NR	0.9
	Jamwal (2018)[39]	↓/↔ (LFAPQ, HLM)	0.8[c]	0.9[c]	NR
	El-Khateeb *et al*. (2021a)[48]	↔ (QTAP, HLM)	NR	NR	1.1[e]
	El-Khateeb *et al*. (2021b)[42]	↓ (QTAP, HLM)	NR	NR	0.4[c,d]
CYP2C18	El-Khateeb *et al*. (2021a)[48]	↓ (QTAP, HLM)	NR	NR	0.5[e]
	El-Khateeb *et al*. (2021b)[42]	↓ (QTAP, HLM)	NR	NR	0.5[c]
CYP2C19	Fisher *et al*. (2009)[96,a]	↓[b] (WB, HLM)	1.5	0.8	0.7
CYP2D6	Fisher *et al*. (2009)[96,a]	↓[b] (WB, HLM)	0.8	0.6	0.7
	Jamwal (2018)[39]	↔ (LFAPQ, HLM)	0.8[c]	0.9[c]	NR
	El-Khateeb *et al*. (2021a)[48]	↓ (QTAP, HLM)	NR	NR	0.6[e]
CYP2E1	Orellana *et al*. (2006)[61,a]	↑/↔ (WB, HLM)	1.1	1.5	NR
	Fisher *et al*. (2009)[96,a]	↓[b] (WB, HLM)	1.1	0.4	0.6
	Jamwal (2018)[39]	↔ (LFAPQ, HLM)	0.9[c]	1.0[c]	NR
	El-Khateeb *et al*. (2021a)[48]	↑ (QTAP, HLM)	NR	NR	1.3
	El-Khateeb *et al*. (2021b)[42]	↓ (QTAP, HLM)	NR	NR	0.4[c,d]
CYP2J2	El-Khateeb *et al*. (2021a)[48]	↓ (QTAP, HLM)	NR	NR	0.6
CYP3A4	Fisher *et al*. (2009)[96,a]	↓[b] (WB, HLM)	1.3	0.8	0.8
	Jamwal *et al*. (2018)[30]	↓ (LFAPQ, HLM)	0.5[d]	0.5[d]	NR
	Jamwal (2018)[39]	↓ (LFAPQ, HLM)	0.5[c]	0.6[c]	NR
	El-Khateeb *et al*. (2021a)[48]	↓ (QTAP, HLM)	NR	NR	0.5[e]
	El-Khateeb *et al*. (2021b)[42]	↓ (QTAP, HLM)	NR	NR	0.2[c,d]
CYP3A5	Jamwal (2018)[39]	↓ (LFAPQ, HLM)	0.5[c]	0.6[c]	NR
CYP4F2	Jamwal (2018)[39]	↔ (LFAPQ, HLM)	0.7[c]	0.9[c]	NR
	El-Khateeb *et al*. (2021a)[48]	↔ (QTAP, HLM)	NR	NR	1.0[e]
	El-Khateeb *et al*. (2021b)[42]	↓ (QTAP, HLM)	NR	NR	0.2[c,d]
CYP4F3	Jamwal (2018)[39]	↔ (LFAPQ, HLM)	1.0[c]	1.0[c]	NR
CYP4F11	Kakehashi *et al*. (2017)[97]	↔ (RQP, FFPE)	NR	NR	1.0
	Jamwal (2018)[39]	↔ (LFAPQ, HLM)	1.0[c]	1.0[c]	NR

(Continued)

Table 2 (Continued)

Enzyme	Study	Trend change in protein levels with increasing disease severity (technique, sample type)	Fold-change in protein levels relative to control by disease group		
			NAFL	NASH w/o cirrhosis	NASH w/ cirrhosis
CYP27A1	Jamwal (2018)[39]	↔ (LFAPQ, HLM)	1.0[c]	1.0[c]	NR
CYP51A1	Kakehashi *et al*. (2017)[97]	↔ (RQP, FFPE)	NR	NR	1.0
	Jamwal (2018)[39]	↔ (LFAPQ, HLM)	0.8[c]	0.9[c]	NR
Non-cytochrome P450 metabolizing enzymes					
ADH1A	Jamwal (2018)[39]	↔ (LFAPQ, CF)	0.9[c]	1.1[c]	NR
ADH1B	Kakehashi *et al*. (2017)[97]	↓/↔ (RQP, FFPE)	NR	NR	0.8[d]
	Jamwal (2018)[39]	↔ (LFAPQ, CF)	0.8[c]	0.9[c]	NR
ADH1C	Kakehashi *et al*. (2017)[97]	↓/↔ (RQP, FFPE)	NR	NR	0.8[d]
	Jamwal (2018)[39]	↔ (LFAPQ, CF)	0.8[c]	0.8[c]	NR
ADH4	Kakehashi *et al*. (2017)[97]	↓/↔ (RQP, FFPE)	NR	NR	0.8[d]
	Jamwal (2018)[39]	↔ (LFAPQ, CF)	0.8[c]	0.9[c]	NR
ADH5	Jamwal (2018)[39]	↔ (LFAPQ, CF)	0.9[c]	1.0[c]	NR
ADH6	Jamwal (2018)[39]	↔ (LFAPQ, CF)	0.8[c]	0.9[c]	NR
ALDH1A1	Jamwal (2018)[39]	↔ (LFAPQ, CF)	0.9[c]	0.9[c]	NR
ALDH1B1	Jamwal (2018)[39]	↔ (LFAPQ, CF)	1.0[c]	1.1[c]	NR
ALDH2	Kakehashi *et al*. (2017)[97]	↓/↔ (RQP, FFPE)	NR	NR	0.8[d]
	Jamwal (2018)[39]	↔ (LFAPQ, CF)	0.8[c]	0.9[c]	NR
AOX1	Jamwal (2018)[39]	↔ (LFAPQ, CF)	0.9[c]	0.9[c]	NR
CES1	Jamwal (2018)[39]	↔ (LFAPQ, CF)	1.0[c]	1.0[c]	NR
	El-Khateeb *et al*. (2021a)[48]	↓ (QTAP, HLM)	NR	NR	0.6[e]
	El-Khateeb *et al*. (2021b)[42]	↓ (QTAP, HLM)	NR	NR	0.2[c,d]
CES2	Jamwal (2018)[39]	↔ (LFAPQ, CF)	0.8[c]	0.9[c]	NR
	El-Khateeb *et al*. (2021a)[48]	↔ (QTAP, HLM)	NR	NR	0.8[e]
FMO3	El-Khateeb *et al*. (2021a)[48]	↔ (QTAP, HLM)	NR	NR	0.8[e]
	El-Khateeb *et al*. (2021b)[42]	↓ (QTAP, HLM)	NR	NR	0.3[c,d]
FMO5	El-Khateeb *et al*. (2021a)[48]	↔ (QTAP, HLM)	NR	NR	0.9[e]
	El-Khateeb *et al*. (2021b)[42]	↓ (QTAP, HLM)	NR	NR	0.2[c,d]
GSTA1	Kakehashi *et al*. (2017)[97]	↓ (RQP, FFPE)	NR	NR	0.7[d]
	Jamwal (2018)[39]	↑/↔ (LFAPQ, CF)	0.9[c]	1.3[c]	NR
GSTA2	Jamwal (2018)[39]	↔ (LFAPQ, CF)	0.8[c]	0.8[c]	NR
GSTP1	Jamwal (2018)[39]	↔ (LFAPQ, CF)	1.1[c]	1.0[c]	NR
GSTZ1	Jamwal (2018)[39]	↔ (LFAPQ, CF)	0.8[c]	0.9[c]	NR
SULT1A1	Hardwick *et al*. (2013)[98,a]	↔ (WB, CF)	1.3[d]	0.4	0.7
	Jamwal (2018)[39]	↔ (LFAPQ, CF)	0.7[c,d]	0.8[c]	NR
SULT1A2	Jamwal (2018)[39]	↔ (LFAPQ, CF)	0.8[c]	0.9[c]	NR
SULT1B1	Jamwal (2018)[39]	↔ (LFAPQ, CF)	0.9[c]	1.0[c]	NR
SULT1C4	Hardwick *et al*. (2013)[98,a]	↑ (WB, CF)	2.3	3.4[d]	6.6[d]
SULT2A1	Hardwick *et al*. (2013)[98,a]	↓ (WB, CF)	2.0	0.6	0.6
	Jamwal (2018)[39]	↔ (LFAPQ, CF)	0.7[c,d]	0.8[c]	NR
UGT1A1	Hardwick *et al*. (2013)[98,a]	↔ (WB, HLM)	1.2	1.0	1.0
	Jamwal (2018)[39]	↔ (LFAPQ, HLM)	1.0[c]	0.9[c]	NR
	El-Khateeb *et al*. (2021a)[48]	↑ (QTAP, HLM)	NR	NR	1.5[e]
	El-Khateeb *et al*. (2021b)[42]	↓ (QTAP, HLM)	NR	NR	0.4[c]

(Continued)

Table 2 (Continued)

Enzyme	Study	Trend change in protein levels with increasing disease severity (technique, sample type)	Fold-change in protein levels relative to control by disease group		
			NAFL	NASH w/o cirrhosis	NASH w/ cirrhosis
UGT1A4	Jamwal (2018)[39]	↔ (LFAPQ, HLM)	0.8[c]	0.8[c]	NR
	El-Khateeb *et al*. (2021a)[48]	↓ (QTAP, HLM)	NR	NR	0.7[e]
UGT1A6	Hardwick *et al*. (2013)[98,a]	↓ (WB, HLM)	1.1	0.6	0.5[d]
	Jamwal (2018)[39]	↔ (LFAPQ, HLM)	1.1[c]	1.1[c]	NR
	El-Khateeb *et al*. (2021a)[48]	↔ (QTAP, HLM)	NR	NR	1.0[e]
	El-Khateeb *et al*. (2021b)[42]	↓ (QTAP, HLM)	NR	NR	0.3[c]
UGT1A7	Jamwal (2018)[39]	↔ (LFAPQ, HLM)	1.1[c]	1.0[c]	NR
UGT1A9	Hardwick *et al*. (2013)[98,a]	↔ (WB, HLM)	2.5	0.9	0.7
	Jamwal (2018)[39]	↔ (LFAPQ, HLM)	1.0[c]	0.8[c]	NR
	El-Khateeb *et al*. (2021a)[48]	↔ (QTAP, HLM)	NR	NR	0.9[e]
	El-Khateeb *et al*. (2021b)[42]	↓ (QTAP, HLM)	NR	NR	0.4[c,d]
UGT2B4	Jamwal (2018)[39]	↔ (LFAPQ, HLM)	0.9	0.9	NR
	El-Khateeb *et al*. (2021a)[48]	↔ (QTAP, HLM)	NR	NR	0.9[e]
	El-Khateeb *et al*. (2021b)[42]	↓ (QTAP, HLM)	NR	NR	0.3[c,d]
UGT2B7	Kakehashi *et al*. (2017)[97]	↔ (RQP, FFPE)	NR	NR	1.0
	Jamwal (2018)[39]	↔ (LFAPQ, HLM)	0.9[c]	1.1[c]	NR
	El-Khateeb *et al*. (2021a)[48]	↓ (QTAP, HLM)	NR	NR	0.7[e]
	El-Khateeb *et al*. (2021b)[42]	↓ (QTAP, HLM)	NR	NR	0.3[c,d]
UGT2B10	Hardwick *et al*. (2013)[98,a]	↔ (WB, HLM)	0.9	0.7	0.8
	Jamwal (2018)[39]	↔ (LFAPQ, HLM)	0.9[c]	1.1[c]	NR
UGT2B15	El-Khateeb *et al*. (2021b)[42]	↓ (QTAP, HLM)	NR	NR	0.5[c]
UGT2B17	Kakehashi *et al*. (2017)[97]	↔ (RQP, FFPE)	NR	NR	1.0
	El-Khateeb *et al*. (2021a)[48]	↔ (QTAP, HLM)	NR	NR	0.9[e]

See **Supplementary Data 1** for detailed clinical information of control/comparator and disease groups. See **Supplementary Data 2** for reported study values and further information on the fold-change calculations between control/comparator and disease groups.

ADH, alcohol dehydrogenase; ALDH, aldehyde dehydrogenase; AOX, aldehyde oxidase; CES, carboxylesterase; CF, cytosolic fraction; FFPE, formalin-fixed paraffin-embedded; FMO, flavin-containing monooxygenase; GST, glutathione S-transferase; HLM, human liver microsomes; LFAPQ, label-free absolute protein quantification; NAFL, nonalcoholic fatty liver (i.e., simple steatosis); NAFLD, nonalcoholic fatty liver disease (encompasses NAFL and NASH); NASH, Nonalcoholic steatohepatitis; NR, not reported; QTAP, quantitative targeted absolute proteomics; RQP, relative quantitative proteomics; SULT, sulfotransferase; UGT, uridine 5′-diphospho-glucuronosyltransferase; w/, with; w/o, without; WB, Western blot.

↔ Indiscernible or no trend based on subjective assessment; ↑ Increasing trend based on subjective assessment; ↓ Decreasing trend based on subjective assessment.

[a]Protein level data extracted using WebPlotDigitizer version 4.2. (https://apps.automeris.io/wpd/). [b]Statistically significant trend ($P < 0.05$) observed when compared across control and disease groups using a nonparametric test for trend as reported in the referenced study. [c]Data were transformed using a scaling factor and are reported as pmol/g liver. [d]Statistically significant difference ($P < 0.05$) compared with control/comparator group as reported in the referenced study. [e]No statistical testing to evaluate significant differences performed/reported in the referenced study.

was noted in the extent of fold-changes compared to controls. Only two studies[30,39] used a BMI-matched non-NAFLD cohort as the comparator for evaluation of changes in DME abundance. This is particularly important for accurate interpretation of proteomic data due to apparent negative and positive correlations between BMI and activity of CYP3A4 and CYP2E1, respectively.[40] Genotype information for DMEs was not available, except for *CYP3A4* and *CYP3A5* in one of the studies.[30] The extent of reported NAFLD-associated changes in protein levels of CYP3A5, CYP2D6, CYP2A4, CYP2B6, CYP2C9, CYP2C19, UGT1A1, and UGT1A3 could be confounded by polymorphisms in genes encoding the corresponding DME.

NAFLD-MEDIATED ALTERATIONS IN HEPATIC DRUG TRANSPORTERS

Drug transport proteins (hereafter "transporters") facilitate cellular entry ("uptake") and exit ("efflux") of exogenous and endogenous compounds and are often a rate-limiting factor in the clearance of small molecule drugs and metabolites. Transporters also play key roles in DDIs. Although transporters are ubiquitously expressed, this section will focus on NAFLD-mediated changes in hepatic drug transporters (see the section on NAFLD-Mediated Alterations in Extrahepatic Enzymes, and Transporters for a review of NAFLD and extrahepatic transporters). Drug transporter mRNA and protein levels do not necessarily correlate,

and they can be altered differently by disease.[41] Therefore, only NAFLD-mediated alterations in transporters at the protein level are reported in **Table 3** and are discussed throughout this section.

Protein levels of hepatic transporters are often impacted in CLD,[10,12] and can have significant implications for PBPK modeling.[42] With NAFLD prevalence increasing globally, studies investigating the impact of this disease and its progression on transporters have emerged over the past decade. From 2011 to 2021, several published studies characterized the impact of NAFLD on protein levels for 18 hepatic drug transport proteins (**Table 3**). In general, a progressive decrease in protein levels of uptake transporters (e.g., organic anion transporting polypeptides [OATPs], sodium taurocholate cotransporting polypeptide [NTCP]) and increase in efflux transporter abundances (e.g., breast cancer resistance protein [BCRP], multidrug resistance-associated proteins [MRPs], and multidrug resistance protein (MDR) 1/P-glycoprotein [P-gp]) seems to occur in NAFLD. These changes could explain, in part, the observed clinical pharmacokinetic profiles of some drugs in NAFLD[17] (see the section on Impact of NAFLD on Clinical Pharmacokinetics). However, more clinical data, including detailed characterization of disease stage, are needed to correlate observed progressive trends in protein levels with *in vivo* transporter function.

Across studies, most transporters show good consistency in directional changes in protein levels, and trends often correspond with NAFLD progression. Apparent interstudy discrepancies were only observed for MRP2 and OATP1B1, which may be due to differences in study design or methodology. Vildhede *et al.*[17] used quantitative targeted absolute proteomics (QTAP) to assess MRP2 and OATP1B1 abundance in NAFLD patients, whereas other studies utilized Western blot (WB)[43–45] or immunohistochemistry (IHC) staining[46] techniques to assess protein abundance. QTAP and/or IHC studies reported decreases in MRP2 and OATP1B1 protein in NASH, whereas most WB studies reported increased abundance of these proteins. In contrast, Clarke *et al.*[45] reported no change in OATP1B1 levels measured by WB. Notably, QTAP and WB studies reporting decreased or no change in MRP2 and/or OATP1B1 levels used membrane fractions (MFs) for protein assessment,[17,45] whereas WB studies showing increased abundance of MRP2 and/or OATP1B1 used whole cell lysates.[43,45,47] This may suggest a NAFLD-mediated increase in accumulation of MRP2 and OATP1B1 in other cellular compartments not retained in MFs. Another potential reason for interstudy differences could be the use of different non-NAFLD control groups[41] (for further discussion, see the section on Current Challenges and Future Directions of Predicting Pharmacokinetics and Pharmacological Outcomes in NAFLD). Although transporter data measured in human liver microsomes from patients with NASH with cirrhosis are available (i.e., bile salt export pump [BSEP], MRP2/3, OAT2, and OATP2B1),[42,48] human liver microsomes typically contain proteins bound to various cellular MFs,[49] and it is unknown how this may compare to crude MFs or whole cell lysate typically used for mass spectrometry analysis of drug transporters. Therefore, these data have been omitted from **Table 3**.

Hepatic transporters must be trafficked to the plasma membrane for proper hepatocellular uptake or efflux of substrates. Therefore, total transport protein abundance may not always align with *in vivo* transporter function. In liver disease, cellular trafficking and subsequent localization of hepatic drug transporters can be significantly altered.[12] Intracellular retention of MRP2 was reported in NASH liver samples relative to controls,[43,46,50] which correlates with observed clinical pharmacokinetic data[50–52] and altered N-linked glycosylation of MRP2 in NAFLD.[43,45] N-linked glycosylation is required for proper protein folding, plasma membrane localization, and function of many transporters.[53] While various post-translational modifications ([PTMs]; e.g., glycosylation, phosphorylation, and ubiquitination) can impact transporter regulation and function,[53] only N-linked glycosylation has been investigated thus far in NAFLD[43,45] (**Table 3**). Clarke *et al.*[45] showed that global downregulation of genes involved in N-glycan biosynthesis corresponded with NAFLD progression and that the unglycosylated forms of OATP1B1/1B3/2B1, MRP2, and NTCP were increased. Although no IHC data currently exist for NTCP and OATPs in NAFLD, altered trafficking mechanisms and localization of OATPs have been reported in other diseases,[54] suggesting the potential for mislocalization in NAFLD. Localization, but not N-glycosylation, of BCRP, P-gp, and MRP3 have been investigated in NAFLD with no observed alterations associated with disease progression.[43] Notably, other transporters with known N-glycosylation sites (e.g., organic solute transporter alpha/beta [OSTα/β], and MRP4) have yet to be studied in NAFLD and warrant investigation. Phosphorylation, a regulator of solute carrier (SLC) transporter function,[53] has not been studied in NAFLD despite data suggesting that changes in phosphorylation of other proteins occur with NAFLD disease progression.[55] Recent advances in phosphoproteomics offer potential promise to address this knowledge gap.

To summarize, several factors including PTMs can impact transporter function and may be altered in NAFLD, but only properly localized protein can likely be considered functional for the purposes of IVIVE-PBPK modeling. Protein levels alone may not be sufficient to accurately predict clinical transporter function or inform PBPK model parametrization. Adjustment factors for proteomic data to account for "functional" proteins present on the plasma membrane could be developed to improve modeling predictions.

NAFLD-MEDIATED ALTERATIONS IN HEPATIC NUCLEAR RECEPTORS

Nuclear receptors (NRs) play important roles in regulation of DMEs and transporters and consequently influence pharmacokinetics. In addition, NRs are involved in regulation of lipid and glucose metabolism as well as inflammation and fibrosis pathways. As such, NR dysregulation contributes to the pathogenesis of NAFLD by impacting the integrated control of energy metabolism by the gut-liver-adipose axis. Drugs targeting NRs are being developed to treat NAFLD. Altered expression of hepatic NRs involved in drug enzyme/transporter alterations in NAFLD patients is discussed in the **Supplementary Text** and summarized in **Supplementary Table S1**.

Table 3 NAFLD-mediated alterations in protein levels of human hepatic drug transporters

Transporter	Study	Trend change in protein levels with increasing disease severity (technique, sample type)	Mean fold-change in total protein levels relative to control (Q1–Q3/±SD)			Trend change in glycosylated fraction (technique)[a]	Observed changes in cellular localization (technique)		
			NAFL	NASH w/o cirrhosis	NASH w/ cirrhosis		NAFL	NASH w/o cirrhosis	NASH w/cirrhosis
BCRP	Hardwick *et al*. (2011)[43,c]	↑ (WB, WCL)	0.9 (0.8–2.7)	4.3 (1.5–6.6)	2.6 (2.0–5.9)	NR	Membrane localized, no change from control (IHC)	Membrane localized, no change from control (IHC)	Membrane localized. Staining more prominent than NASH w/o cirrhosis and NAFL (IHC)
BSEP	Okushin *et al*. (2016)[46]	↓ (IHC, FFPE section)	NR	NR	NR	NR	Unclear. Authors discuss progressive decrease in protein staining intensity with increasing NAS score. No analysis or discussion of changes in cellular localization (IHC).		
	Vildhede *et al*. (2020)[17]	↔/↓ (QTAP, MF)	1.0 (NR)	0.8 (NR)[b]		NR	NR	NR	NR
MRP1	Hardwick *et al*. (2011)[43,c]	↑ (WB, WCL)	4.1 (2.3–11.1)[d]	11.1 (4.9–19.9)[d]	11.9 (4.1–24.0)[d]	NR	NR	NR	NR
MRP2	Hardwick *et al*. (2011)[43]	↑[f] (WB, WCL)	NR	NR	NR	↓ (WB, WCL)	Membrane localized, no change from control (IHC)	Membrane localized, no change from control (IHC)	Internalized relative to control (IHC)
	Canet *et al*. (2015)[50]	NR	NR	NR	NR	NR	Membrane localized, no change from control (IHC)	Internalized relative to control (IHC)[b]	
	Okushin *et al*. (2016)[46]	↓ (IHC)	NR	NR	NR	NR	Unclear. Authors discuss progressive decrease in protein staining intensity with increasing NAS score. No analysis or discussion on changes in cellular localization (IHC)		
	Clarke *et al*. (2017)[45]	↑[f] (WB, WCL)	NR	NR	NR	↓ (WB, WCL)	NR	NR	NR
	Vildhede *et al*. (2020)[17]	↓ (QTAP, MF)	1.2 (NR)	0.7 (NR)[b]		NR	NR	NR	NR
MRP3	Hardwick *et al*. (2011)[43,c]	↑ (WB, WCL)	0.7 (0.5–1.4)[d]	1.9 (1.1–2.6)[d]	2.1 (1.4–3.7)[d]	NR	Membrane localized, no change from control (IHC)	Membrane localized, no change from control. Staining more prominent than NAFL and control (IHC)	Membrane localized, no change from control. Staining more prominent than NAFL and control (IHC). Localized primarily to fibrotic areas (IHC)
	Canet *et al*. (2015)[50,c]	↑ (WB, WCL)	0.6 (0.4–1.3)	2.1 (1.2–2.6)[b,e]		NR	NR	NR	NR
	Vildhede *et al*. (2020)[17]	↑ (QTAP, MF)	2.2 (NR)[e]	1.7 (NR)[b,e]		NR	NR	NR	NR

(Continued)

Table 3 (Continued)

Transporter	Study	Trend change in protein levels with increasing disease severity (technique, sample type)	Mean fold-change in total protein levels relative to control (Q1–Q3/±SD)			Trend change in glycosylated fraction (technique)[a]	Observed changes in cellular localization (technique)		
			NAFL	NASH w/o cirrhosis	NASH w/ cirrhosis		NAFL	NASH w/o cirrhosis	NASH w/cirrhosis
MRP4	Hardwick *et al.* (2011)[43,c]	↑ (WB, WCL)	7.3 (1.0–13.5)[d]	28.0 (11.4–125.4)[d]	25.9 (15.5–59.1)[d]	NR	NR	NR	NR
MRP5	Hardwick *et al.* (2011)[43,c]	↑ (WB, WCL)	3.0 (2.3–5.1)[d]	11.2 (8.1–12.9)[d]	9.2 (6.3–14.0)[d]	NR	NR	NR	NR
MRP6	Hardwick *et al.* (2011)[43,c]	↑ (WB, WCL)	7.9 (6.2–12.0)[d]	32.3 (26.1–37.7)[d]	27.8 (22.0–38.6)[d]	NR	NR	NR	NR
NTCP	Aguilar-Olivos *et al.* (2015)[99,c]	↓ (WB, WCL)	NR	0.2 ± 0.1[e,g]		NR	NR	NR	NR
	Clarke *et al.* (2017)[45]	↓[f] (WB, MF)	NR	NR	NR	↓ (WB, MF)	NR	NR	NR
	Vildhede *et al.* (2020)[17]	↓ (QTAP, MF)	0.8 (NR)	0.7 (NR)[b,e]		NR	NR	NR	NR
OAT2	Vildhede *et al.* (2020)[17]	↓ (QTAP, MF)	0.9 (NR)	0.7 (NR)[b,e]		NR	NR	NR	NR
OAT7	Vildhede *et al.* (2020)[17]	↓ (QTAP, MF)	1.6 (NR)[e]	0.8 (NR)[b]		NR	NR	NR	NR
OATP1B1	Clarke *et al.* (2014)[47,c]	↑ (WB, WCL)	0.8 (0.7–1.4)	4.4 (2.6–6.9)[b,e]		NR	NR	NR	NR
	Clarke *et al.* (2017)[45]	↔[f] (WB, MF)	NR	NR	NR	↓ (WB, MF)	NR	NR	NR
	Vildhede *et al.* (2020)[17]	↓ (QTAP, MF)	1.0 (NR)	0.7 (NR)[b,e]		NR	NR	NR	NR
OATP1B3	Clarke *et al.* (2014)[47,c]	↓ (WB, WCL)	0.9 (0.2–6.3)	0.03 (< 0.01–0.05)[b,e]		NR	NR	NR	NR
	Clarke *et al.* (2017)[45]	↑/↔[f] (WB, MF)	NR	NR	NR	↓ (WB, MF)	NR	NR	NR
	Vildhede *et al.* (2020)[17]	↓ (QTAP, MF)	0.8 (NR)	0.4 (NR)[b,e]		NR	NR	NR	NR
OATP2B1	Clarke *et al.* (2014)[47,c]	↔ (WB, WCL)	0.8 (0.7-1.2)	0.8 (0.7–1.2)[b]		NR	NR	NR	NR
	Clarke *et al.* (2017)[45]	↔[f] (WB, MF)	NR	NR	NR	↓ (WB, MF)	NR	NR	NR
	Vildhede *et al.* (2020)[17]	↓ (QTAP, MF)	0.9 (NR)	0.6 (NR)[b,e]		NR	NR	NR	NR
OCT1	Vildhede *et al.* (2020)[17]	↔ (QTAP, MF)	1.4 (NR)	1.0 (NR)[b]		NR	NR	NR	NR
OSTα	Malinen *et al.* (2018)[100,c]	↑ (WB, MF)	NR	NR	2.9 ± 0.7	NR	NR	NR	Apparent membrane localization with some intracellular staining. Control showed negligible staining overall (IF).

(Continued)

Table 3 (Continued)

Transporter	Study	Trend change in protein levels with increasing disease severity (technique, sample type)	Mean fold-change in total protein levels relative to control (Q1–Q3/±SD)			Trend change in glycosylated fraction (technique)[a]	Observed changes in cellular localization (technique)		
			NAFL	NASH w/o cirrhosis	NASH w/ cirrhosis		NAFL	NASH w/o cirrhosis	NASH w/cirrhosis
OSTβ	Malinen *et al.* (2018)[100,c]	↑ (WB, MF)	NR	NR	11.0 ± 7.5[e]	NR	NR	NR	Apparent membrane localization. Control showed weak intracellular/ membrane staining (IF).
P-gp	Hardwick *et al.* (2011)[43,c]	↑ (WB, WCL)	1.2 (0.9-1.8)[d]	2.1 (1.4-5.6)[d]	1.8 (1.6-3.1)[d]	NR	Membrane localized, no change from control (IHC)	Membrane localized. Staining more prominent than steatosis and control (IHC)	Membrane localized. Staining more prominent than steatosis and control (IHC)
	Vildhede *et al.* (2020)[17]	↑ (QTAP, MF)	1.8 (NR)[e]	1.3 (NR)[b]		NR	NR	NR	NR

See **Supplementary Data 1** for detailed clinical information of control/comparator and disease groups. See **Supplementary Data 2** for reported study values and further information on the fold-change calculations between control/comparator and disease groups.

BCRP, breast cancer resistance protein; BSEP, bile salt export pump; FFPE, formalin-fixed paraffin-embedded; HLM, human liver microsomes; IF, immunofluorescence; IHC, immunohistochemistry; MF, membrane fraction; MRP, multidrug resistance-associated protein; NAFL, nonalcoholic fatty liver; NAFLD, nonalcoholic fatty liver disease (encompasses NAFL and NASH); NAS, nonalcoholic fatty liver disease activity score; NASH, nonalcoholic steatohepatitis; NR, not reported; NTCP, sodium/taurocholate cotransporting polypeptide; OAT, organic anion transporter; OATP, organic anion transporting polypeptide; OCT, organic cation transporter; OST, organic solute transporter; P-gp, p-glycoprotein; QTAP, quantitative targeted absolute proteomics; Q1-Q3, quartile 1–quartile 3; SD, standard deviation; w/, with; w/o, without; WB, western blot; WCL, whole cell lysate; ±, with and without.

↔ Indiscernible or no trend based on subjective assessment; ↑ Increasing trend based on subjective assessment; ↓ Decreasing trend based on subjective assessment.

[a]Glycosylated fraction (qualitative assessment) = amount of glycosylated protein/(amount of glycosylated protein + amount of unglycosylated protein). [b]Patient population comprises mix of NASH ± cirrhosis. [c]Protein level data extracted using WebPlotDigitizer (https://apps.automeris.io/wpd/). [d]Statistically significant trend ($P < 0.05$) observed when compared across control and disease groups using a nonparametric test for trend as reported in the referenced study. [e]Statistically significant difference ($P < 0.05$) compared to control/comparator group as reported in the referenced study. [f]Qualitative assessment of "total protein"; Western blot was comprised of separate bands for glycosylated vs. hemi/unglycosylated protein. Densitometry data for each band was normalized to control separately and could not be reliably added together to obtain "total protein" amounts. [g]Cirrhosis status NR.

NAFLD-MEDIATED ALTERATIONS IN EXTRAHEPATIC ENZYMES AND TRANSPORTERS

NAFLD is associated with various extrahepatic, especially metabolic, alterations.[2] Both NAFLD and the metabolic syndrome involve dysregulated signaling between adipose tissue and the liver[20] (see the section on Microphysiologic Hepatic Changes Associated with NAFLD). The resultant systemic abnormalities (e.g., insulin resistance, IL-6, and TNF-α elevations) impact other organs, potentially leading to extrahepatic manifestations, such as T2DM, cardiovascular disease, and CKD, which are all strongly associated with NAFLD.[19,20] Associations of additional extrahepatic diseases (e.g., colorectal cancer, endocrinopathies, and osteoporosis) with NAFLD are suspected, but require further investigation.[20]

Studies on extrahepatic changes in xenobiotic-relevant enzymes, transporters, and NRs in NAFLD patients and its associated disorders are scarce. Extrahepatic ADME alterations in animal models of NAFLD have been studied to a wider extent and are summarized in the **Supplementary Text**.

Although the majority of key DMEs are predominantly expressed in the liver, other organs, including the intestines, kidneys, and lungs, have well-established contributions to drug metabolism. Extrahepatic alterations in CYPs, SULTs, UGTs, and other DMEs in NAFLD could substantially impact xenobiotic exposure, efficacy, and toxicity and require investigation in humans.

Data on extrahepatic transporter regulation in NAFLD are limited, but the first liquid chromatography-tandem mass spectrometry-based proteomic analysis of renal transporter alterations in patients with NAFLD has been reported.[56] During progression from healthy to NASH, trends of kidney transporter abundance fold-changes were observed for apical sodium–dependent bile acid transporter (ASBT) (1.7to 5.3 pmol/mg protein), BCRP (0.15 to 0.06 pmol/mg protein), organic cation transporter (OCT) 2 (157 to 17 pmol/mg protein), MRP3 (0.42 to 0.24 pmol/mg protein), equilibrative nucleoside transporters (ENT) 1; (28.5 to 10.8 pmol/mg protein), and concentrative nucleoside transporters (CNT) 3 (0.19 to 0.09 pmol/mg protein). Fold-changes for organic anion transporter (OAT) 4 abundance from healthy (1.72 pmol/mg protein) to NAFL, and to NASH, were ~ 0.45 (0.78 pmol/mg protein) and ~ 0.27 (0.47 pmol/mg protein), respectively. These observed kidney transporter alterations are likely compensatory in nature. Generally, there is growing support for the hypothesis that transporters (and DMEs) communicate and undergo adaptive changes to maintain homeostasis of circulating molecules.[57] Changes in hepatic disposition of compounds due to liver disease, and the reduction of estimated glomerular filtration rate in NASH,[32] may both play a role in these observed renal transporter alterations.

Information on extrahepatic alterations of NRs, particularly those involved in the regulation of drug disposition (e.g., PXR, CAR, and FXR) in NAFLD is limited and are briefly discussed in the **Supplementary Text**.

The combination of liver-specific and extrahepatic disturbances in ADME processes can have profound effects on xenobiotic disposition in NAFLD and its associated extrahepatic disorders. Although many ADME-related knowledge gaps in human NAFLD need to be filled, it will be extremely challenging to attribute associated extrahepatic ADME alterations solely to NAFLD because of the comorbid disease states with accompanying extrahepatic abnormalities that are often present in this population.

IMPACT OF NAFLD ON CLINICAL PHARMACOKINETICS

Clinical pharmacokinetic studies dedicated to patients with NAFLD and clinical information on drug interactions in this patient population are currently limited and confined to small molecule drugs (**Table 4**). Interspecies differences in protein levels, PTMs, *in vivo* activity, and isoforms (orthologs) of several important DMEs and transporters may limit the clinical translation of animal pharmacokinetic data. Therefore, this section focuses specifically on pharmacokinetic data in humans.

The pharmacokinetics of probe drugs for CYP3A, CYP1A2, CYP2C8, and CYP2C19 were altered in NAFLD patients compared with non-NAFLD cohorts (**Table 4**). Systemic exposure to midazolam, a CYP3A probe, was higher in patients with NAFLD compared with a healthy non-obese cohort (increase in median plasma concentration at 3 hours post-dose to 0.29 from 0.10 ng/mL), consistent with lower protein levels of CYP3A4 in NAFLD.[58] The area under the plasma concentration–time curve (AUC) ratio of 1-hydroxymidazolam to midazolam over 6 hours (metabolite-to-parent ratio) was modestly decreased in pediatric patients with NASH, but not NAFL, compared with healthy individuals.[59] The corresponding metabolite-to-parent ratios in urine samples were highly variable with no consistent trend between healthy and NAFLD populations. However, quantitative data on the abundance of renal UGT enzymes in patients with NASH is lacking. It is worth noting that obesity,[36] T2DM,[30] and pro-inflammatory mediators (e.g., CYP3A suppression by IL-6 in obese patients),[40] in addition to NAFLD, may all contribute to clinically observed changes in the pharmacokinetics of midazolam. Increased CYP2E1 activity observed in patients with NAFLD can be explained primarily by the effect of obesity on abundance and activity of the enzyme.[36] Protein levels of hepatic CYP2E1 in patients with NAFLD did not seem to increase substantially compared with a BMI-matched non-NAFLD control group.[39] The apparent clearance (clearance/oral bioavailability) of chlorzoxazone, a selective CYP2E1 probe, in non-T2DM NASH patients was only slightly higher (24% difference in mean values) than that of healthy individuals with a similar BMI range.[60] This was in concordance with the formation of 6-hydroxychlorzoxazone (catalyzed by CYP2E1), which was not altered in obese patients with NAFL, but was higher in morbidly obese NASH patients compared with a non-NAFLD obese cohort.[61]

In vivo, CYP1A2 activity evaluated by the caffeine breath test was reduced ~ 30% in NASH patients compared with healthy individuals.[62] In contrast, CYP1A2-mediated formation of paraxanthine, the major metabolite (70–80%) of caffeine was not affected by NAFLD in younger patients (12–21 years of age).[59] Steady-state plasma trough concentrations of pioglitazone were 46% higher and hydroxypioglitazone, which is formed predominantly by CYP2C8-mediated hydroxylation of pioglitazone, was 32% lower in NASH patients compared with healthy individuals.[63,64] It is noteworthy that the healthy cohort was younger and possibly not

BMI-matched to the NASH patient population. However, there is no significant association between *in vitro* CYP2C8 activity and body size across a BMI range of 18–63 ekg/m^2.[40] When controlled for *CYP2C9* genotype, the plasma metabolite-to-parent ratio of losartan (a CYP2C9 substrate) was markedly lower in adolescents with NASH compared with a healthy cohort, although the corresponding ratio evaluated using urine samples was not affected by NAFLD disease status.[59] The average therapeutic daily dose of warfarin (S-warfarin), another CYP2C9 substrate, was not significantly different between NAFLD patients with and without cirrhosis compared with non-NAFLD control groups in two separate studies.[65,66] This is in line with nonsignificant changes in protein levels of CYP2C9 (involved in warfarin metabolism) and CYP4F2 (responsible for catabolism of vitamin K and associated with interindividual variability in warfarin response) in patients with non-cirrhotic NAFLD compared with non-NAFLD individuals with similar BMI.[39] Despite observing no change in warfarin daily dose relative to the control group, Zhang *et al.*[66] reported a lower international normalized ratio to warfarin dose ratio in NAFLD (cirrhosis status not reported), suggesting potential NAFLD-mediated alterations in vitamin K regulation or other pharmacodynamic factors that may impact warfarin response. It should be noted, however, that this study did not control for pharmacogenetic factors involved in warfarin response. The potential mechanism(s) of decreased warfarin response in this population require further investigation.

Milk thistle extract contains two major flavonolignans, silybin A and silybin B, both of which undergo extensive glucuronidation by various UGT enzymes (i.e., hepatic UGT1A1 and UGT1A3, intestinal UGT1A8, and UGT1A10).[67] Interestingly, although systemic exposure (AUC) of intact and conjugated silybin A and B was higher in NAFLD patients compared with those in healthy individuals, the fraction metabolized to glucuronides was not significantly different.[68] These data suggest that lower activity of MRP2 and OATP1B1/1B3, and higher MRP3 activity are responsible for changes in hepatic disposition of the silybin glucuronides in NAFLD patients compared with healthy individuals. Similarly, higher observed systemic concentrations of glucuronide metabolites of morphine[52] and acetaminophen in patients with NASH[50] are consistent with decreased MRP2 and increased MRP3 efflux. Systemic exposures of apixaban and rosuvastatin in patients with NAFLD are slightly lower, albeit not significantly different than those in healthy lean individuals.[69] Despite being a CYP3A substrate, apixaban only undergoes minor metabolism by the isoenzymes (fraction metabolized < 0.25) and thus, the NAFLD-associated decrease in CYP3A activity may be attenuated by a presumed increase in intestinal (and hepatic) BCRP and/or P-gp activity. The reason for a lack of significant change in rosuvastatin pharmacokinetics between patients with NAFLD and the non-NAFLD cohort is not clear,[69] but might be related to the counterbalance of increased BCRP-mediated efflux in enterocytes (and hepatocytes) and decreased OATP1B1/1B3-mediated hepatic uptake of rosuvastatin in the NAFLD patient population. However, quantitative proteomics data for BCRP and P-gp in the enterocytes of NAFLD patients has not been published.

Intrahepatic concentrations of metformin, an antidiabetic drug, did not differ between NAFL and NASH patients[70] and appeared to be comparable to that in healthy individuals with two functional *SLC22A1* alleles,[71] consistent with little to no difference in abundance of hepatic OCT1 across the disease and healthy populations.[17] Despite similar concentrations at the site of action (liver), total systemic exposure of metformin in patients with NASH may be higher than that in healthy individuals (hence, potential for increased risk of metformin toxicity) due to the disease-associated reduction in abundance of renal OCT2.[56] Additional investigation is needed to confirm this hypothesis.

CURRENT CHALLENGES AND FUTURE DIRECTIONS OF PREDICTING PHARMACOKINETICS AND PHARMACOLOGICAL OUTCOMES IN NAFLD

The application of IVIVE-linked PBPK methods in pharmacokinetic prediction for drug development and clinical pharmacology/personalized medicine has significantly advanced in the 21st century.[15] As vast clinical data have emerged to inform development of diverse virtual populations, PBPK modeling has become increasingly accepted by regulatory agencies to supplement new drug applications in special populations.[16] However, inherent limitations with the IVIVE-PBPK/PD approach remain for the NAFLD population, particularly at different stages of disease progression.

A major advantage of IVIVE-PBPK is the ability to use scaling factors to translate *in vitro* and *ex vivo* proteomic data to biologically relevant parameters for model input. Many ADME-associated protein studies in NAFLD to date utilize relative (e.g., WB) rather than absolute quantification approaches using liquid chromatography-tandem mass spectrometry-based proteomics. However, quantitative absolute proteomics is becoming increasingly more common for IVIVE of ADME-associated proteins (**Tables 2 and 3**). Many researchers now use membrane-enriched fractions of human tissue for the measurements of ADME-associated proteins.[72] Before these abundance data can be applied in IVIVE-PBPK, several points need to be considered. First, losses that occur during MF enrichment from whole cell lysate need to be accounted for because underestimated abundance will lead to underestimation of the scaled clearance.[73] Hence, the yield of the MF obtained should routinely be reported together with the abundance data. Second, it is well-known that microsomal or membrane protein per gram of tissue exhibits age-dependent variability[74]; therefore, the age of donors should be reported or alternatively the reported abundance should be age-corrected. Third, and particularly relevant for plasma membrane bound proteins like transporters, suitable membrane markers to correct for protein loss during sample preparation must be used. Membrane markers can be organ-specific and suit different purposes, but are currently not standardized.[72] Failure to appropriately account for these factors could prove problematic for accurate assessment of "functional" DMEs and drug transporters.

Immunohistochemical imaging in tandem with proteomic data is a potential approach to quantify properly localized transporters and DMEs. Imaging data also can be used to investigate the heterogeneous histology of NAFLD tissue samples as it pertains to protein

Table 4 Clinical pharmacokinetic studies in NAFLD

Drug	Study	Dosing regimen	PK parameter	Mean fold-change in PK parameter relative to control			Proposed mechanism underlying PK changes[a]
				NAFL	NASH w/o Cirrhosis	NASH w/ Cirrhosis	
Adult population							
DMEs primarily mediate elimination of the drug							
Caffeine	Park *et al.* (2011)[62]	2 mg/kg p.o. × single dose as [3-methyl-^{13}C] caffeine	^{13}C-caffeine breath test value (^{13}C-enrichment of expired CO_2 per 100 mg caffeine)	1.0	0.7[b]	0.4[b]	↓ CYP1A2
Chlorzoxazone	Chalasani *et al.* (2003)[60]	500 mg p.o. × single dose	Apparent clearance (CL/F)	NR	1.2[c]	NR	↑ CYP2E1 (potentially due to obesity, rather than NASH)
	Orellana *et al.* (2006)[61]	500 mg p.o. × single dose	M/P plasma concentration at 2 hr	1.4[c]	3.1[b,d]		
Midazolam	Woolsey *et al.* (2015)[58,e]	100 µg p.o. × single dose	Plasma concentration, 3 hr post-dose	2.7	2.9[b,d]		↓ CYP3A
Pioglitazone[f]	**Control/ Comparator:** Manitpisitkul *et al.* (2014)[64] **NASH Group:** Kawaguchi-Suzuki *et al.* (2017)[63]	**Control/ Comparator:** 30 mg p.o. qday × 8 days **NASH Group:** 30 mg p.o. qday titrated to 45 mg p.o. qday after 2 months	**Control/ Comparator:** Trough plasma concentration ($C_{min,ss}$) at day 8 of drug therapy (steady-state) **NASH Group:** Trough plasma concentration ($C_{min,ss}$) at month 18 of drug therapy (steady-state)	NR	Parent (Pioglitazone) 1.5[g] Metabolite (hydroxypioglitazone) 0.7[b,g]		↓ CYP2C8 ↓ CYP3A
Transporters primarily mediate elimination of the drug							
Apixaban	Tirona *et al.* (2018)[69]	2.5 mg p.o. × single dose	Plasma AUC_{0-12h}	0.9	0.8	NR	↓ CYP3A ↑ BCRP, P-gp
^{99m}Tc-Mebrofenin	Ali *et al.* (2017)[51]	~ 2.5 mCi i.v. bolus × single dose as ^{99m}Tc-mebrofenin	$AUC_{0-300min,blood}$ $AUC_{0-180min,liver}$	NR	$AUC_{0-300min,blood}$: 1.4[b] $AUC_{0-180min,liver}$: 1.6[b]	NR	↓ MRP2, OATP1B1/1B3
Metformin[g]	Sundelin *et al.* (2017)[71,e]; Sundelin *et al.* (2019)[70,e]	**Control/ Comparator**[h]: 188 (range: 109–235) MBq of ^{11}C-metformin i.v. bolus × single dose **NAFLD Group:** 102–222 MBq of ^{11}C-metformin i.v. bolus × single dose	Peak hepatic SUV (g/mL) SUV = concentration (kBq/mL) × weight (g)/dose (kBq)	1.1	1.01[i]		↔ OCT1 ↓ OCT2
Morphine	Ferslew *et al.* (2015)[52]	5 mg i.v. infusion (over 5 min) × single dose	AUC_{0-last}	NR	Parent (Morphine) 0.9 Metabolite (Morphine Glucuronides) 1.6[b]	NR	Parent (Morphine) ↔ UGT2B7 ↔ OCT1 Metabolite (Morphine Glucuronides) ↓ MRP2, OATP1B1/1B3 ↑ MRP3

(Continued)

Table 4 (Continued)

Drug	Study	Dosing regimen	PK parameter	Mean fold-change in PK parameter relative to control			Proposed mechanism underlying PK changes[a]
				NAFL	NASH w/o Cirrhosis	NASH w/ Cirrhosis	
Rosuvastatin	Tirona *et al.* (2018)[69]	5 mg p.o.	Plasma $AUC_{0\text{-}12h}$	0.9	0.7	NR	↓ MRP2, OATP1B1/1B3 ↑ BCRP
Silymarin [silybin A (SA) and silybin B (SB)]	Schrieber *et al.* (2008)[68]	480 mg p.o. × single dose	Plasma $AUC_{0\text{-}24h}$		Parent SA 1.2[j] Parent SB 3.7[j] Sum Total Silymarin flavonoligans 3.3[j,k]		Parents (SA + SB) ↔ UGT1A1, 1A3, 1A8, 1A10 Metabolites (SA + SB Conjugates, other Silymarin flavonoligans) ↓ MRP2, OATP1B1/1B3 ↑ MRP3
Pediatric population							
DMEs primarily mediate elimination of the drug							
Losartan	Li *et al.* (2017)[59]	25 mg p.o. × single dose	M/P plasma and urinary $AUC_{0\text{-}6h}$	Plasma: 1.1[l] Urine: 0.9[l]	Plasma: 0.3[l] Urine: 0.9[l]	NR	↔ or ↓ CYP2C9
Midazolam	Li *et al.* (2017)[59]	2 mg p.o. × single dose	M/P plasma and urinary $AUC_{0\text{-}6hr}$	Plasma: 0.9[l] Urine: 0.8[l]	Plasma: 0.6[l] Urine: 1.0[l]	NR	↓ CYP3A
Omeprazole	Li *et al.* (2017)[59]	20 mg p.o. × single dose	M/P plasma and urinary $AUC_{0\text{-}6hr}$	Plasma: 0.4[l] Urine: 0.8[l]	Plasma: 0.2[l] Urine: 0.3[l]	NR	↓ CYP2C19
Transporters primarily mediate elimination of the drug							
Acetaminophen (APAP)	Canet *et al.* (2015)[50]	1 g p.o. × single dose	Plasma $AUC_{0\text{-}4h}$	Parent (APAP) 1.0 Metabolite (APAP-Glucuronide) 0.8 Metabolite (APAP-Sulfate) 0.9	Parent (APAP) 0.8 Metabolite (APAP-Glucuronide) 1.5 Metabolite (APAP-Sulfate) 0.5	NR	Parent (APAP) ↔ UGT1A1, 1A6, 1A9, 2B15 Metabolite (APAP-Glucuronide) ↓ MRP2 ↑ MRP3

See **Supplementary Data 1** for detailed clinical information of control/comparator and disease groups. See **Supplementary Data 2** for reported study values and further information on the fold-change calculations between control/comparator and disease groups.
AUC, area under the plasma concentration–time curve; BCRP, breast cancer resistance protein; CL, clearance; Css, concentration at steady-state; CYP, cytochrome P450; F, bioavailability; g, grams; hr, hour; i.v., intravenous; kBq, kilobecquerels; MBq, megabecquerels; mCi, millicuries; mg, milligrams; min, minutes; MRI, magnetic resonance imaging; MRP, multidrug resistance-associated protein; M/P, metabolite-to-parent ratio; NAFL, nonalcoholic fatty liver (i.e., simple steatosis); NAFLD, nonalcoholic fatty liver disease (encompasses NAFL and NASH); NAS, NAFLD activity score; NASH, nonalcoholic steatohepatitis; NR, not reported; OATP, organic anion transporting polypeptide; OCT, organic cation transporter; PK, pharmacokinetics; p.o., oral dosing; qday, once daily dosing; SUV, standardized uptake value; UGT, uridine 5′-diphospho-glucuronosyltransferase; w/, with; w/o, without; ±, with and without
↔ Indiscernible or no trend based on subjective assessment; ↑ Increasing trend based on subjective assessment; ↓ Decreasing trend based on subjective assessment. [a]The direction of the arrow depicts trend of changes in activity (and/or abundance) of the metabolizing enzymes and transporters in patients with NAFLD compared with that of control/comparator group. [b]Statistically significant difference ($P < 0.05$) from control/comparator group as reported in the referenced study. [c]Comparison was made between the NAFLD patient population and BMI-matched non-NAFLD individuals. [d]Cirrhosis status NR.
[e]Protein level data extracted using WebPlotDigitizer (https://apps.automeris.io/wpd/). [f]Pharmacokinetic data for control/comparator group and NAFLD patients was taken from different clinical studies with similar demographics (see **Supplemental Data 1 and 2**). [g]Calculated based on dose-normalized trough concentrations ($C_{min,ss}$/daily dose). [h]Only includes *SLC22A1* wild-type homozygotes as reported in study. [i]Patient population comprises mix of NASH ± cirrhosis. [j]Patient population comprises mix of NAFL, NASH w/o cirrhosis and NASH w/ cirrhosis. [k]A sum of parent and conjugates (glucuronide and sulfate conjugates). [l]Only includes individuals with *CYP2C9* reference sequence as reported in study.

abundance or cellular localization. These data may provide a better understanding of zonal changes in DMEs and transporters in NAFLD, which could have significant implications from a pharmacokinetic modeling standpoint.[75] PTMs of proteins also may be key to informing appropriate "functional" parameterization of DMEs and transporters. For example, N-linked glycosylation impacts transporter localization in NAFLD (see the section on NAFLD-Mediated Alterations in Hepatic Drug Transporters). Other PTM changes may occur during NAFLD progression and warrant investigation. Despite data suggesting an altered phosphoproteome in NAFLD[55] and the known impact of phosphorylation on DME[76,77] and transporter[53] function, this remains an understudied area.

Development of a membrane protein per gram liver scaling factor for the NAFLD population would be particularly useful

Table 5 Recommendations to advance physiologically based modeling for patients across stages of NAFLD progression from NAFL to NASH

Knowledge gaps
- **Physiologic Data** – Obtain accurate ADME-relevant physiologic characteristics (e.g., hepatocellularity, plasma protein levels, liver weight, blood flow) across histological stages of NAFLD progression and in the presence of concomitant diseases (e.g., T2DM)
- **Localization of ADME Proteins** – Obtain detailed cellular-level (e.g., intracellular vs. membrane localized for transporters) and tissue-level (e.g., zonal distribution for transporters and DMEs) data to determine changes across stages of NAFLD progression
- **Post-translational Modifications (PTMs) in ADME Proteins** – Determine impact of NAFLD-mediated alterations in PTMs (e.g., N-linked glycosylation, phosphorylation, ubiquitination) on drug disposition
- **Functional ADME Data** – Conduct clinical PK studies with metabolic and transporter probe substrates to determine alterations across stages of NAFLD progression; *in vivo* clinical imaging data would allow for additional insight into tissue drug concentrations[51,70,71]
- **Pediatric Population** – Determine interplay of ontogeny and NAFLD-mediated impact on ADME-associated proteins

In vitro methodology refinement
- **Plasma Membrane-Specific Protein Quantification for Transporters** – Refine tissue membrane extraction techniques that specifically target plasma membrane bound protein; apply biotinylation methods to human tissue samples; conduct quantitative colocalization analysis using IHC/IF
- ***In vitro* Models** – Develop *in vitro* models that simulate stages of NAFLD progression in humans and facilitate IVIVE

Clinical Studies
- **Control Groups** – Standardize control/comparator groups across studies (e.g., age-/BMI-matched) and report detailed demographic information (e.g., comorbidities, medication history)
- **Control Group Adjustments** – Develop adjustment factors to account for variability across different control/comparator groups (e.g., healthy donor vs. carcinoma adjacent resection) in proteomic studies[41] to avoid inaccurate fold-change estimates
- **NAFLD Groups** – Utilize well-defined histologic data (e.g., fibrosis, steatosis content) and clinically validated scoring system(s) (NAS, FIB-4) and report detailed patient demographic information

Considerations for IVIVE-PBPK/PD Modeling
- **Sample Preparation for Scaling Abundance Data** – Utilize appropriate sample preparation technique (WCL vs. microsomal, S9, or MF) based on research aims
- **Scaling Factors** – Develop accurate organ-specific scaling factors across NAFLD stages for microsomal protein per gram (MPPG) tissue (e.g., MPPGL for liver) and S9 protein per gram (S9PPG) tissue (e.g., S9PPGL for liver) for DMEs; membrane protein per gram (MePPG) tissue (e.g., MePPGL for liver) for transporters; cytosolic protein per gram (CPPG) tissue (e.g., CPPGL for liver) and hepatocytes per gram liver (HPGL) for DMEs and transporters
- **Virtual Populations** – Develop populations for specific subgroups across stages of NAFLD progression (e.g., NASH w/o cirrhosis) to avoid assumptions of homogeneity. Demographic factors associated with higher risk of NAFLD (e.g., ethnicity, socioeconomic status, environment) also should be considered to develop a virtual population representative of real-world NAFLD patients.

ADME, absorption, distribution, metabolism, excretion; DME, drug metabolizing enzyme; FIB-4, fibrosis-4 score; IF, immunofluorescence; IHC, immunohistochemistry; IVIVE, *in vitro*-to-*in vivo* extrapolation; MF, membrane fraction; NAFL, nonalcoholic fatty liver; NAFLD, nonalcoholic fatty liver disease (encompasses NAFL and NASH); NAS, nonalcoholic fatty liver disease activity score; NASH, nonalcoholic fatty liver disease; PBPK/PD, physiologically based pharmacokinetic/pharmacodynamic; PK, pharmacokinetic; PTM, post-translational modification; S9, 9,000 g force supernatant of liver homogenate; T2DM, type 2 diabetes mellitus; WCL, whole cell lysate; w/o, without.

for accurately translating quantitative MF proteomic data to the organ level. Microsomal protein per gram liver scaling factors have been developed for NASH cirrhosis[31] (**Table 1**) and can drastically change unscaled observed differences in DME abundance relative to control[42,48] (**Table 2**). This example highlights the importance of using scaling factors to more accurately inform PBPK model parameterization. Assumptions about the impact of NAFLD and its progression on physiologic parameters and scaling factors can be made but require validation with clinical data. More clinical studies are also needed to assess ADME-relevant physiologic characteristics, such as organ blood flow and plasma protein levels at each stage of NAFLD with validated histological/disease scoring (e.g., NAS,[6] fibrosis scoring systems[3]) and healthy, BMI-matched controls for comparison.[41] Most ADME-relevant clinical NAFLD studies utilize qualitative categorization of histological features of NAFLD (i.e., simple steatosis, NASH with or without cirrhosis) rather than reporting data from standardized clinical scoring systems. Often, different control/comparator groups are used, which can limit cross-study comparisons (**Tables 1-4**, **Supplementary Table S1**, **Supplemental Data 1**). To date, initial PBPK models with a specific focus have adequately described clinical data in NAFLD and NASH cohorts, but sample sizes have been limited.[17,18] A major challenge for PBPK modeling of NAFLD is the development of a virtual population(s) to accurately encompass this progressive and heterogenous disease in adult and pediatric patients, where detailed physiologic measurements and proteomic scaling factors across the NAFLD spectrum are even more scarce. Notably, ancestral groups disproportionately impacted by NAFLD (e.g., Hispanics) are vastly under-represented in available studies to date, limiting the generalizability of virtual population-based simulations to real-world patients. Furthermore, a paucity of reliable data on physiologic characteristics and ontogeny of DMEs (and transporters) in children with NAFLD limits the extrapolation of PBPK modeling from adult to younger patients with NAFLD. A histological scoring system for NAFLD has been clinically validated for use in pediatrics[6] and should be utilized for further study of ADME-associated proteins and physiologic parameters in this population. Refined physiologic (**Table 1**) and functional ADME and toxicity data will be crucial to advance PBPK/PD modeling for patients with NAFLD of all ages. Recommendations to further advance and improve applications of physiologically based modeling in NAFLD are summarized in **Table 5**.

Due to feasibility and practicality, most clinical pharmacokinetic studies only measure systemic drug concentrations. Systemic drug concentrations, however, do not always reflect intracellular/organ drug concentrations, particularly in the presence of active

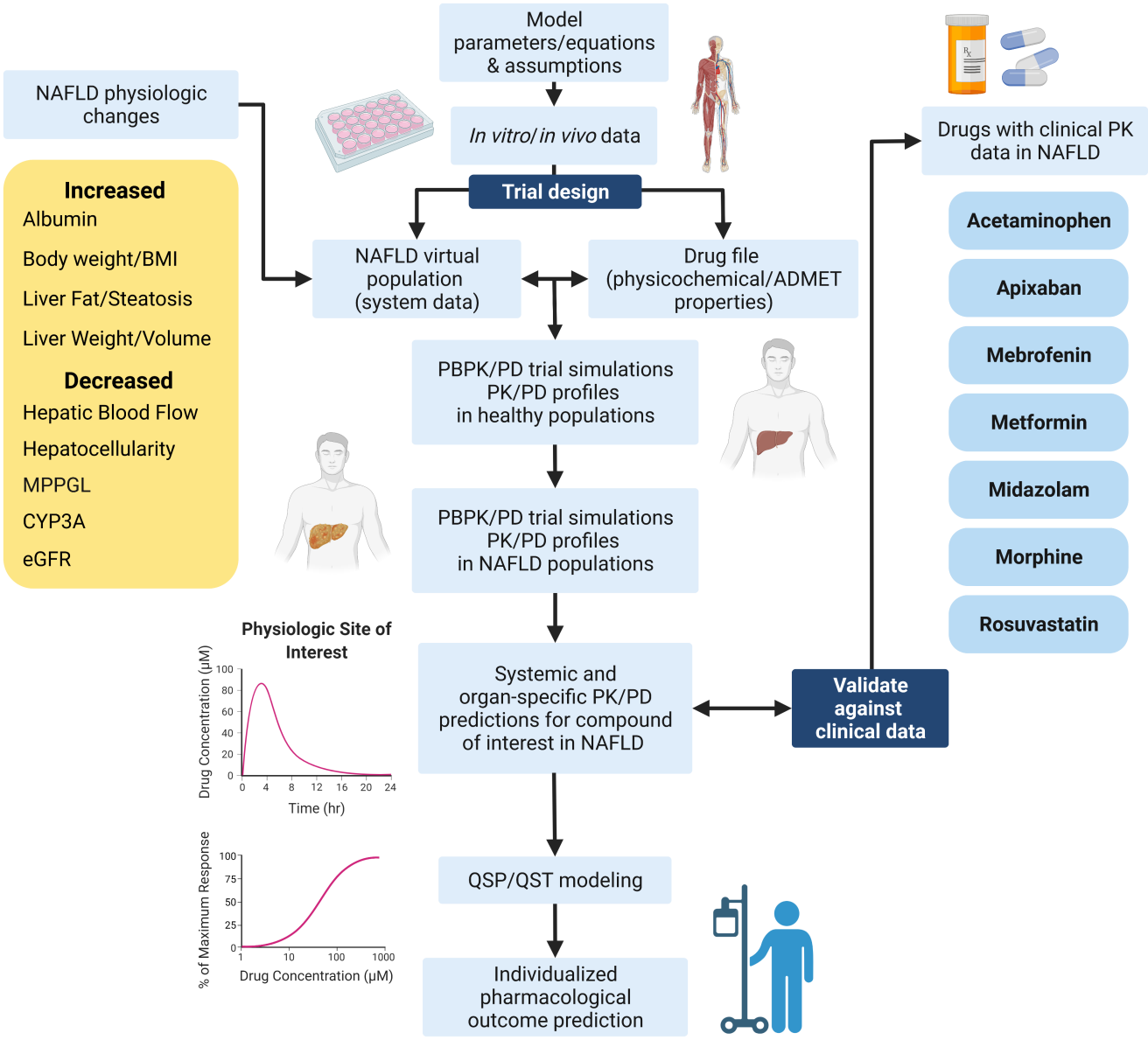

Figure 2 PBPK/PD modeling is a powerful computational tool used to predict drug concentrations and effects at a physiologic site of interest, which can be leveraged to inform QSP/QST models to predict pharmacological outcomes in complex disease states, such as NAFLD. Development of a PBPK/PD model requires complex differential equations with *a priori* assumptions, along with detailed physiologic and drug data acquired from *in vitro* and/or *in vivo* sources. Known physiologic changes in NAFLD (see **Figure 1** for more detail) coupled with physiochemical data for a drug can be used to perform trial simulations in a virtual NAFLD and healthy population using various software programs. If the appropriate parameters are available, both systemic and organ-specific concentrations and effects can be predicted. However, trial simulation output/predictions for drugs with overlapping pathways of metabolism and/or transport must be validated using clinical data from the same population. Predicted PK/PD data at physiologic sites of interest can then be used to inform QSP/QST modeling to predict pharmacological outcomes.
Created with BioRender.com. ADMET, absorption, distribution, metabolism, excretion, toxicity; BMI, body mass index; CYP, cytochrome P450; eGFR, estimated glomerular filtration rate; MPPGL, microsomal protein per gram of liver; NAFLD, nonalcoholic fatty liver disease (encompasses NAFL and NASH); NDA, new drug application; PD, pharmacodynamics; PK, pharmacokinetics; PBPK/PD, physiologically based pharmacokinetic/pharmacodynamic; QSP, quantitative systems pharmacology; QST, quantitative systems toxicology.

transport and metabolism. Because many small molecule drugs must partition into tissue and cells to be detoxified or exert their pharmacologic effect, estimates of intracellular drug concentrations are essential for accurately predicting pharmacological outcomes. An additional utility of PBPK modeling is the ability to simulate intra-organ and intracellular drug concentrations in the absence of verifiable clinical data.[15,16] Development and application of NAFLD virtual populations could provide valuable insight into how intracellular/organ drug concentrations may be altered, even in the absence of systemic differences. For example, Tirona *et al.*[69] reported no difference in rosuvastatin and apixaban plasma concentrations in patients with NAFLD relative to healthy

controls (Table 4), but potential alterations in hepatic drug concentrations of these drugs are unknown. Because both rosuvastatin and apixaban are hepatically metabolized and have therapeutic targets that originate in the liver, potential alterations in intrahepatic concentrations could have significant clinical implications.

In vivo clinical imaging data, although costly and time-consuming to obtain, can be used to validate PBPK model predictions and provide important insight into intra-organ drug concentrations in clinical scenarios. Two studies thus far have utilized *in vivo* imaging for studying intrahepatic concentrations in NAFLD[51,70] (Table 4). *In vivo* imaging approaches could be especially useful in evaluating intra-organ/target site exposure and drug disposition in the presence of multiple transporter alterations across different stages of NAFLD progression and warrant future application (Table 5).

Tissue concentrations of drugs simulated by PBPK modeling or observed in clinical imaging studies can be coupled with PD modeling to predict drug effects at a physiologic site of action. Drug exposure and effects predicted by PBPK/PD modeling also can be utilized in QSP and QST modeling (Figure 2). QSP/QST modeling involves a bottom-up approach similar to PBPK modeling, and includes mathematical representation of cellular physiology, disease pathophysiology, mechanisms of efficacy/toxicity with a representation of specific targets, and relevant biomarkers to quantitatively understand and predict the pharmacologic and/or toxicologic effects of small molecule and biologic drugs. Tissue concentrations of drugs in patients with NAFLD predicted by PBPK modeling are particularly valuable in QSP/QST modeling to predict efficacy and/or toxicity of drugs indicated for patients with NAFLD, given that many of the treatment targets for this disease reside in the liver. Time-resolved drug concentrations simulated by PBPK modeling can be combined with target engagement data (e.g., half-maximal inhibitory concentration and half-maximal effective concentration) to predict treatment or toxicity responses at cellular, organ, and/or whole-body levels in QSP/QST models, as reported previously.[78] NAFLD disease progression and/or organ toxicity due to drug effects (e.g., drug-induced liver injury) could subsequently influence organ clearance and pharmacokinetics of drugs. A PBPK/PD model fully integrated into QSP/QST models can allow prediction of dynamic pharmacokinetic-pharmacodynamic interactions over the course of long-term pharmacotherapy,[79] which is critical for medications indicated for chronic conditions, such as NAFLD and metabolic syndrome-related comorbidities.

Endogenous biomarkers, liquid biopsies, and even probe cocktails, may prove useful in clinical studies to characterize changes in protein levels of DMEs and transporters across stages of disease progression from NAFL to NASH.[80] For example, the endogenous biomarker coproporphyrin I, a substrate of OATP1B1 and MRP2, potentially could be used to assess hepatic transporter function in patients with NAFLD.[81] Bile acids may be useful to gauge NAFLD disease progression and serum bile acid profiling in clinical pharmacokinetic studies may provide important information.[22]

Numerous genetic and dietary rodent models have been used widely to mimic NAFLD for preclinical drug testing (reviewed by Soret[82] and Dietrich[83]) and to predict altered drug ADME and pharmacokinetics,[44,47] but limitations exist in translation of preclinical data to patients with NAFLD. The search for cellular and *in vivo* models that replicate clinically observed changes in NAFLD has intensified as this highly heterogenous and complex disease has emerged as a global epidemic.[82] Ideally, data generated from these model systems would inform PBPK/PD model development. Culturing primary human hepatocytes (PHHs) or hepatic cell lines in various formats with fatty acid-enriched media is a common approach to simulate NAFLD *in vitro*, but PHH data revealed that this is not sufficient to replicate the hepatocellular lipidome of patients with NASH.[84] Immortalized hepatic cell lines typically have altered metabolic functions that limit *in vivo* translation. Advances in hepatic spheroids and organoids using PHH or hepatic stem cells appear promising, and liver- and gut-liver-on-a-chip technologies may markedly increase the ability to mimic NAFLD *in vitro*.[82] However, these methods/techniques require further improvement to recapitulate the progression of this chronic disease. Furthermore, the high complexity and cost of these more advanced models may limit widespread use for *in vitro* pharmacokinetic studies. Important considerations in the development of these *in vitro* models include: (1) appropriate validation of the model (e.g., are the relevant DMEs and transporters expressed, properly localized, and functional; are relevant regulatory mechanisms functional); (2) determination of which substrates can be used to generate data for modeling; and (3) identification of specific probe compounds or probe cocktails for routine use as controls to confirm that the model is functioning appropriately when applied to new compounds.

CONCLUSIONS

Despite inherent challenges, IVIVE-PBPK is a useful method for informing pharmacokinetic predictions, particularly in special populations, such as NAFLD, where limited clinical pharmacokinetic data exist. Virtual populations would be valuable for capturing ADME-relevant physiologic changes and informing PBPK/PD and QSP/QST simulations for drugs across different histological stages of NAFLD (Figure 2). However, reliable system and drug data to build the virtual populations are needed. As summarized in this review, system data (e.g., data regarding ADME-related proteins) are emerging from patients with NAFLD but knowledge gaps remain (Table 5). There is a clear need for a better understanding of altered drug absorption and disposition beyond the liver in the highly comorbid NAFLD population where polypharmacy adds significant complexity to appropriate medication management. The ultimate goal of achieving optimal pharmacotherapy in patients with NAFLD will require accurate predictions of clinically relevant pharmacological outcomes (e.g., DDIs, drug efficacy, and toxicity) for this population during drug development and in clinical practice. PBPK/PD modeling coupled with QST/QSP applications offer great potential to achieve this goal.

SUPPORTING INFORMATION

Supplementary information accompanies this paper on the *Clinical Pharmacology & Therapeutics* website (www.cpt-journal.com).

FUNDING

This work was funded in part by the Certara Simcyp 2020/2021 Grant and Partnership Scheme (GPS) awarded to Dr. William A. Murphy and a Simulations Plus Postdoctoral Fellowship awarded to Dr. Jeffry Adiwidjaja.

CONFLICT OF INTEREST

Dr. Murphy is supported by the Certara Simcyp 2020/2021 Grant and Partnership Scheme (GPS). Dr. Adiwidjaja is supported by a Simulations Plus Postdoctoral Fellowship. Drs. Yang, Beaudoin, and Siler are employees of DILIsym Services Division, Simulations Plus Inc. Dr. Spires is an employee of Simulations Plus. Dr. Neuhoff is an employee of Certara UK Ltd. All other authors have no conflicts of interest to declare.

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