Representative Rodent Models for Renal Transporter Alterations in Human Nonalcoholic Steatohepatitis

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Rodent Models for Renal Transporter Alteration in Human NASH

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Abbreviations: American lifestyle induced obesity syndrome (ALIOS), atherogenic (Athero), breast cancer resistance protein (BCRP), copper transporter (CTR), equilibrative nucleoside transporter (ENT), fast food thioacetamide (FFDTH), methionine-choline-deficient (MCD), multidrug and toxin extrusion (MATE), multidrug resistance-associated protein (MRP), multidrug resistance protein (MDR), nonalcoholic fatty liver disease (NAFLD), nonalcoholic steatohepatitis (NASH), organic anion transporter (OAT), organic anion transporting peptides (OATP), organic cation transporters (OCT), organic cation uptake transporter (OCTN), peptide transporter (PEPT), sodium taurocholate cotransporting polypeptide (NTCP), sodium-glucose cotransporter (SGLT), urate transporter (URAT)
ABSTRACT

Alterations in renal elimination processes of glomerular filtration and active tubular secretion by renal transporters can result in adverse drug reactions. Nonalcoholic steatohepatitis (NASH) alters hepatic transporter expression and xenobiotic elimination, but until recently, renal transporter alterations in NASH were unknown. This study investigates renal transporter changes in rodent models of NASH to identify a model that recapitulates human alterations. Quantitative protein expression by surrogate peptide LC-MS/MS on renal biopsies from NASH patients were used for concordance analysis with rodent models, including methionine-choline-deficient (MCD), atherogenic (Athero) or control rats; Lepr\textsuperscript{db/db} MCD (db/db), C57BL/6J fast food thioacetamide (FFDTH), American lifestyle induced obesity syndrome (ALIOS), or control mice. Demonstrating clinical similarity to NASH patients, db/db, FFDTH, and ALIOS showed decreases in GFR by 76, 28, and 24%. Organic anion transporter 3 (OAT3) showed an upward trend in all models except the FFDTH (from 3.20 to 2.39 pmol/mg protein), making the latter the only model to represent human OAT3 changes. OAT5, a functional ortholog of human OAT4, significantly decreased in db/db, FFDTH, and ALIOS (from 4.59 to 0.45, 1.59, and 2.83 pmol/mg protein, respectively), but significantly increased for MCD (1.67 to 4.17 pmol/mg protein), suggesting the mouse models are comparable to human for these specific transport processes. These data suggest variations in rodent renal transporter expression are elicited by NASH and the concordance analysis enables appropriate model selection for future pharmacokinetic studies based on transporter specificity. These models provide a valuable resource to extrapolate the consequences of human variability in renal drug elimination.
SIGNIFICANCE STATEMENT

Rodent models of nonalcoholic steatohepatitis that recapitulate human renal transporter alterations are identified for future transporter specific pharmacokinetic studies to facilitate the prevention of adverse drug reactions due to human variability.
INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) and its irreversible progressive form, nonalcoholic steatohepatitis (NASH), are among the top etiologies of liver disease that lead to liver dysfunction (Asrani et al., 2019). Due to the rising obesity epidemic, and advancements in vaccinations and treatments for viral hepatitis, NAFLD and NASH are predicted to surpass viral hepatitis as the leading cause of liver disease in the United States (Ocker, 2020; Younossi et al., 2020). The global prevalence of NAFLD is approximately 25% and rises to an alarming 90% in obese patients (Asrani et al., 2019; Polyzos et al., 2019; Ocker, 2020; Younossi et al., 2020). NASH, however, is grossly underestimated at 1.5-6.5% due to ascertainment bias, limited imaging sensitivity, and invasive diagnosis with a liver biopsy (Anstee et al., 2013; Younossi, 2018). Nonetheless, new waitlist registrants for liver transplants in the United States with NASH increased by 170% from the years 2004 to 2013 (Wong et al., 2015). The increasing frequency of NASH is concerning given that NASH is known to elicit alterations in drug disposition by the disruption of hepatic drug transporters, leading to potential adverse drug reactions (Hardwick et al., 2011; Dzierlenga and Cherrington, 2018; Vildhede et al., 2020).

A general decrease in expression of hepatic basolateral uptake transporters, a functional decrease in canalicular efflux transporter multidrug resistance-associated protein 2 (MRP2), and an increase in hepatic basolateral efflux transporters observed in NASH promotes shifts in the predicted pharmacokinetics of numerous xenobiotics. This functional alteration results in plasma retention of parent compounds and reduces biliary excretion of metabolites, which inherently places the kidney at risk of exposure to higher concentrations of xenobiotics (Hardwick et al., 2011; Hardwick et al., 2014; Dzierlenga and Cherrington, 2018; Vildhede et al., 2020). However, there is little information concerning adaptions in renal transporter expression that may compensate for increased xenobiotic exposure the kidney might encounter during NASH. Renal physiological changes during NASH have been postulated due to the altered pharmacokinetics of adefovir, with a decrease in glomerular filtration rate (GFR) and increase in tubular secretion in rodents, and a clinical observation of decreased estimated GFR in NASH patients (Targher et al., 2010; Laho et al., 2016). Considering the number of therapeutics that depend on renal elimination and the increasing prevalence of NASH, it is imperative that alterations of xenobiotic distribution and elimination be investigated to prevent potential toxicities.
To investigate the alteration of xenobiotic pharmacokinetics due to hepatic inflammation and renal compensation, rodent models that reflect changes in transporter expression observed in human NASH, along with associated modifications of renal function, must be identified. Therefore, this study uses well-established rodent models of NASH for characterization and correlation to human alterations in kidney physiology. Biochemical enzymatic function tests, as well as transcriptional and translational expression profiles are analyzed for each model to support the hypothesis of renal physiological changes during times of hepatic stress in NASH. These findings facilitate the identification of substrate dependent rodent models for future pharmacokinetic studies that exemplify the alterations in renal transporter expression of NASH patients. This study provides a resource of representative rodent model selection for studies that will improve therapeutic treatment and reduce potential adverse drug reactions with medications that have a misrepresented disposition due to renal xenobiotic transporter alterations during NASH.

MATERIALS AND METHODS

Materials

Iohexol was purchased from TCI, Inc. (Portland, OR). Sodium deoxycholate and dithiothreitol (DTT) were purchased from Sigma-Aldrich (St. Louis, MO). Ammonium bicarbonate was purchased from Oakwood Chemical (Estill, SC). Iodoacetamide (IAA) was purchased from Biorad (Hercules, CA). All LC-MS grade solvents were purchased from Fisher Scientific (Pittsburgh, PA).

Animals

Male 8-week-old Sprague Dawley rats were obtained from Charles Rivers Laboratories (Wilmington, MA). Male 5-week-old C57BL/6J and B6BKS(D)-Lepr<db>J (db/db) mice were obtained from Jackson Laboratories (Bar Harbor, ME). All animals were acclimated for at least 1 week in a University of Arizona Association for Assessment and Accreditation of Laboratory Animal Care-certified animal facility with a standard 12-hour light/dark schedule with standard chow and water ad libitum. All housing and experimental procedures were in accordance with National Institutes of Health guidelines and are compliant with the University of Arizona Institutional Animal Care and Use Committee. To induce NASH, Sprague Dawley rats (n = 6) were fed a methionine- and choline-deficient diet (MCD; #518810; Dyets, Inc., Bethlehem, PA) or atherogenic diet (Athero; D12492; Research Diets, Inc., New Brunswick, NJ) for 8 weeks. An MCD diet was also fed to db/db mice (n = 6) for 4 weeks to induce NASH. To model NASH in
the C57BL/6J mice (n = 6), an amino acid defined fast food diet (FFDTH; 1919974-280; Test Diet) was fed with 75 mg/kg intraperitoneal (IP) injections of thioacetamide (Sigma-Aldrich, St. Louis, MO) thrice weekly for 8 weeks, or the American Lifestyle Induced Obesity Syndrome diet (ALIOS; TD. 130885; Envigo Teklad Diets, Madison, WI) for 25 weeks. Control Sprague Dawley rats (n = 6) and C57BL/6J mice (n = 6) were fed a choline-sufficient and iron supplemented L-AA defined diet (#518753; Dyets, Inc., Bethlehem, PA) for 8 and 6 weeks, respectively.

**Pathology**

Liver and kidney tissues were collected and weighed with portions fixed in 10% neutral buffered formalin for pathological analysis and the remaining tissue was snap frozen in liquid nitrogen and stored in a -80°C freezer until analysis. Hematoxylin and eosin (H&E) and Masson’s trichrome stains were performed on the fixed tissue to assess pathology. Hepatic and renal scoring systems were used to assess tissue injury. Scoring was graded as 0, no significant lesions (0%); 1, minimal (<10%); 2, mild (10-25%); 3, moderate (25-40%); 4, marked (40-50%); 5, severe (>50%) for renal and liver lesions. Renal lesions included necrosis, degeneration, regeneration, tubule dilation, epithelial cell loss, and glomerular change. Liver lesions included lipids accumulation, necrosis, apoptosis, inflammation, fibrosis, and biliary hyperplasia. Overall injury scores were evaluated according to the discretion of the toxicological study pathologist to confirm the presence of NASH in each disease model and absence of NASH in control models.

**Clinical Chemistry**

At the conclusion of dietary feeding, a loading dose of 20 mg/kg iohexol prepared in sterile saline was administered by an intravenous (IV) injection for rats and IP injection for mice. Tail vein (~500µL) and submental (~30 µL) blood were collected from each rat and mouse, respectively, at 30 and 90 minutes after IV injection of iohexol. Animals were euthanized by CO₂ asphyxiation at 120 minutes post injection of iohexol. Terminal blood was collected via cardiac puncture and all blood samples were centrifuged at 2,000 X g for 10 minutes at 4°C for plasma extraction. A 300 µL aliquot of terminal plasma was sent to the University of Arizona Health Science Center Animal Care Clinical Pathology Department to perform a full comprehensive clinical chemistry panel for kidney and liver function tests. Alkaline phosphatase
(ALP), alanine aminotransferase (ALT), glucose, creatinine, and blood urea nitrogen (BUN) concentrations were determined.

To determine the GFR of each model, the clearance of iohexol was determined at 30- and 90-minutes post iohexol injection, adapted from an algorithm previously published by Turner et al. (Turner et al., 2017). Iohexol was quantified in plasma using LC-MS/MS and adjusted for body weight as previously published by Jilek et al. (Jilek et al., 2021a). Briefly, a liquid-liquid extraction was performed with 1 mL of 50 ng/mL internal standard d5-iohexol in acetonitrile (ACN) vortexed with 5 µL of plasma and centrifuged at 2,000 x g for 30 minutes at 4°C. Eight hundred microliters of the supernatant was transferred to a new plate and dried down before reconstituting with 100 µL of the mobile phase (95:5 water/ACN + 0.1% formic acid). A Shimadzu LC-20AD liquid chromatography system was used to inject 2 µL of the reconstituted analytes onto a 50 mm X 2.1 mm Luna® Omega Polar C18 column with 1.6 µm particles and separated by a binary flow gradient of water + 0.1% formic acid and ACN + 0.1% formic acid. Under multiple reaction monitoring (MRM), the analyte constituents were detected using a Sciex QTrap® 4500+ mass spectrometer with electrospray ionization (ESI) in positive mode. The analyte transitions used for quantification were 821.7 → 803.6 for iohexol and 826.7 → 808.7 for d6-iohexol. Analyst MultiQuant™ (Sciex) was used to quantify the analytes and plasma iohexol clearance was calculated using AUC (CL = AUC0-∞/Dose).

Transcriptional Expression

RNA from approximately 80 mg of kidney tissue was isolated per manufacturer protocols with Invitrogen TRIzol RNA Isolation Reagent (ThermoFisher Scientific, Waltham, MA) and the RNA pellet was reconstituted in 500 µL of sterile nuclease-free water. The RNA concentration was determined using a NanoDrop 2000 UV-vis spectrophotometer (ThermoFisher Scientific, Waltham, MA). Reactions were prepared with biological replicates containing 10 µL of ReadyScript cDNA synthesis kit (Sigma-Aldrich, Burlington, MA), 1 µL of TaqMan gene expression assay Gapdh (ThermoFisher Scientific, Waltham, MA), 1 µL of TaqMan gene expression assay of respective gene, 2 µL of isolated RNA, and 8 µL of nuclease-free water were added to respective wells of a 96-well reaction plate. To examine technically variability, a no template control was included for every gene on respective plates and the reference gene, Gapdh, was added to each reaction as a VIC-MGB fluorescent probe while respective genes were FAM-MGB.
fluorescent probes. cDNA preparation and RNA quantification were performed on an ABI StepOnePlus Real-Time PCR System (Waltham, MA) using recommended cycling modes of ReadyScript cDNA synthesis kit directly followed by TaqMan Fast Advanced Master Mix User Guide cycling recommendations. Fold change (FC) was determined by the \(2^{-\Delta\Delta C_{\text{t}} G} \) method using biological replicates and a control gene per reaction for solute carriers (SLC) and ATP-binding cassette transporters (ABC) (Livak and Schmittgen, 2001).

**Protein Expression**

Insoluble membrane protein fractions were isolated and digested from kidney tissue using a method adapted from a published protocol by Jilek *et al* (Jilek et al., 2021b). Approximately 100 mg of tissue was homogenized in 1 mL of buffer containing 10 mM Tris-HCl (pH 7.4), 10 mM NaCl, 1.5 mM MgCl₂, 1 mM PMSF, and 1X protease inhibitor per 50mL of water. Following homogenization, cells were further disrupted by douncing then centrifuged at 1,000 \(\times\) g for 2 minutes at 4\(^\circ\) C directly followed by 8,000 \(\times\) g for 10 minutes. The S9 fraction was collected and centrifuged at 100,000 \(\times\) g for 60 minutes at 4\(^\circ\) C. The insoluble protein pellet was reconstituted in 100 \(\mu\)L of 10 mM tris-HCl (pH 8.0) and stored at -80\(^\circ\) C until protein quantification by Pierce™ Protein BCA Assay Kit (Thermo Fisher, Waltham, MA). Samples were normalized to 300 \(\mu\)g in water and incubated with denaturing buffer (3.7% sodium deoxycholate, 6 mmol/L DTT, and 100 mmol/L ammonium bicarbonate) for 5 minutes at 95\(^\circ\) C, followed by alkylation with 15 mmol/L IAA in the dark for 30 minutes. The IAA reaction was quenched with 20 mmol/L DTT. Protein was digested by sequencing-grade trypsin (Promega, Inc., Madison, WI) using a 1:100 enzyme to substrate ratio overnight at 37\(^\circ\) C. Heavy isotope labeled amino acid (\(^{13}\)C/\(^{16}\)N) peptide internal standard cocktail with 0.4% formic acid (Table 1) was added to acidify the reaction for protein precipitation. The samples were centrifuged at 16,000 \(\times\) g for 15 minutes and the supernatant was collected before undergoing strong cation exchange solid phase extraction (SPE) per Waters, Inc. cartridge protocol. The peptides were eluted off the columns with 200 \(\mu\)L of 60:40 water/ACN with 2% ammonium hydroxide, followed by 200 \(\mu\)L of ACN with 2% ammonium hydroxide. Eluent was collected and dried in a speed vac at 60\(^\circ\) C, then reconstituted in 50 \(\mu\)L of 95:5 water/ACN + 0.1% formic acid.

Surrogate peptide protein quantification was performed according to instrumental parameters published by Frost and Jilek *et al.*, with adjusted rodent peptide sequences and transitions (Table
In summary, an Agilent UPLC system was used to inject 10 µL of reconstituted peptides onto a 2.1 mm X 100 mm Waters, Inc. C18 column with 1.8 µm particles with a binary gradient mobile phase (aqueous: water + 0.1% formic acid; organic: 90:10 ACN/water + 0.1% formic acid). Peptides were detected under MRM operated in positive mode ESI on a Sciex QTrap 6500+ mass spectrometer for a minimum of two transitions for each respective protein (Table S1). Analyst Multiquant (version 3, Sciex) was used to integrate internal standards and calibrators (pure standards at 0.01, 0.1, 0.5, and 1 ng) for quantification and all values reported are within limits of quantification respective of each calibration curve. Protein abundance was calculated using Equation 1 which accounts for protein yield following digestion and SPE.

Equation 1: Protein Abundance (pmol/mg Protein) = (Surrogate Peptide (pg)/ Peptide MW (g/mol)) X (1/Peptide Input (mg))

Protein abundance values for human renal transporters by Frost and Jilek et al. were used for concordance analysis to compare rodent and human renal transporter expression for: organic anion transporters (OAT), sodium taurocholate cotransporting polypeptide (NTCP), organic cation transporters (OCT), organic anion transporting peptides (OATP), copper transporter (CTR), sodium-glucose cotransporter (SGLT), organic cation uptake transporter (OCTN), urate transporter (URAT), peptide transporter (PEPT), multidrug resistance-associated protein (MRP), multidrug and toxin extrusion (MATE), equilibrative nucleoside transporter (ENT), multidrug resistance protein (MDR), and breast cancer resistance protein (BCRP) (Frost and Jilek et al., 2022).

Statistical Analysis

GraphPad Prism 9.0 software (San Diego, CA) was used to analyze and graph the data. A one-way analysis of variance with Fisher’s LSD test was performed and is presented as the mean ± standard deviation, with statistical significance defined at p ≤ 0.05. Multiple comparisons were made between respective rodent control and disease model with biological replicates (n = 6). Protein expression concordance analysis across human and rodent models was performed using Equation 2 with effect sizes estimated by Glass’s Δ (Canet et al., 2014).

Equation 2: Δ = \( \bar{x}_1 - \bar{x}_2 \)/Sp

Where \( \bar{x}_1 \) and \( \bar{x}_2 \) are the sample means for two groups and Sp is the pooled standard deviation for both.
RESULTS

Pathology

Hepatic pathology was assessed by an H&E and Masson’s trichrome stain on formalin-fixed, paraffin-embedded (FFPE) tissues. All models except db/db resulted in a significant overall liver pathology score representing the development of NASH relative to respective controls (Figure 1 and Table S2). The renal pathology for each rodent was determined by an H&E stain on FFPE tissues. Since renal pathological lesions that accompany NASH are not well characterized, kidney grading criteria for a variety of lesions included degeneration/vacuolation, lipid accumulation, necrosis, apoptosis, inflammation, hyaline casts, and glomerular changes. There were no changes in any renal lesion category for control or NASH models, apart from mild lipid accumulation in ALIOS (Table S2).

Clinical Chemistry

Clinical chemistry plasma panels and iohexol clearance were used to examine common liver and kidney function tests. All rat models fell within the normal range for ALP (174-589 U/L) except for a significant increase in Athero (374 ± 89.6 U/L) relative to control (248 ± 67.3 U/L). All mouse models were above the normal range of ALP (35-96 U/L) with db/db (189 ± 2.83 U/L) and ALIOS (141 ± 23.9 U/L) having a significant increase relative to control (119 ± 11.8 U/L). ALT significantly increased in MCD (265 ± 104 U/L) and ALIOS (4237 ± 2462 U/L) relative to rat control (29.2 ± 8.59 U/L) and mouse control (128 ± 44.3 U/L), respectively. All models surpassed the normal range of ALT for rats (35-80 U/L) and mice (17-77 U/L) except for rat control. There were no significant increases in glucose for the rat models, which all stayed at or slightly above the normal range (50-160 mg/dL). Interestingly, all mouse models, including control, were elevated above the normal range of glucose (62-175 mg/dL), but the NASH mouse models (db/db 194 ± 26.8 mg/dL; FFDTH 314 ± 81.0 mg/dL; ALIOS 356 ± 110 mg/dL) were significantly decreased from mouse control (450 ± 106 mg/dL) (Figure 2).

All models remained within the normal ranges (rats 10-21 mg/dL; mice 8-33 mg/dL) for BUN, although MCD (19.4 ± 2.06 mg/dL), Athero (17.0 ± 2.40 mg/dL), and ALIOS (29.4 ± 5.71 mg/dL) significantly increased relative to respective controls (rat 12.0 ± 1.33 mg/dL; mouse 20.8 ± 1.59 mg/dL).
There were no changes in creatinine for rat models and all, including control, fell slightly below the normal range (0.5-1 mg/dL). All mouse models fell within the normal range of creatinine (0.2-0.9 mg/dL) with only FFDTH (1.33 ± 0.60 mg/dL) significantly increased relative to control (0.58 ± 0.18 mg/dL). Iohexol clearance for Athero significantly increased to 265% relative to control, however, in the mouse models, iohexol clearance significantly decreased by 76, 28, and 24% relative to control for db/db, FFDTH, and ALIOS, respectively (Figure 2).

Renal Transporter Transcriptional Expression

Significant changes in relative mRNA expression were determined for renal uptake and efflux transporters. There were no changes in basolateral uptake transporters for the rat models relative to control except for an increase in SLC22A1 (OCT1) for MCD (121%) and Athero (134%). The db/db mouse model also increased for SLC22A1 (OCT1), to 115% relative to control. The only other increase in basolateral uptake transporters was for SLC22A8 (OAT3), to 196% for db/db and 128% for ALIOS, relative to control. The decreases observed in mice relative to control were in SLC22A6 (OAT1) by 91% for db/db, SLC22A8 (OAT3) by 26% for FFDTH, and SLC22A2 (OCT2) by 61 and 18% for db/db and FFDTH, respectively (Figure 3A). Apical uptake transporters SLC22A7 (OAT2) and SLCO1A1 (OAT1A1) demonstrated no significant change for the rat models, however, all of the mouse models decreased in SLC22A7 (OAT2) for db/db, FFDTH, and ALIOS by 99.8, 27, and 62% and in SLCO1A1 (OAT1A1) by 98, 78, and 46%, respectively, compared to control (Figure 3B). Apical efflux transporter SLC29A1 (ENT1) and basolateral efflux transporter ABCB1b (MDR1b), were only commercially available for rat and showed no significant changes (Figure 3C and 3D). Additional basolateral efflux transporters, ABCC2 (MRP2), ABCC4 (MRP4), ABCG2 (BCRP), and SLC47A1 (MATE1) did not change relative to rat control for MCD and Athero models. A decrease in ABCC2 (MRP2), ABCG2 (BCRP), and SLC47A1 (MATE1) by 35, 12, and 31% was observed in FFDTH compared to control. A decrease in ABCC2 (MRP2) by 25% was also observed for ALIOS compared to control (Figure 3D).

Protein Expression of Renal Uptake Transporters

Significant changes in protein expression of renal uptake transporters were analysed by LC-MS/MS. MCD showed an upward trend in expression of several uptake transporters, including increases...
in OAT2 (35.7 ± 16.8); OAT3 (8.49 ± 3.80); OAT5 (20.9 ± 6.58); OATP2A1 (0.80 ± 0.30); and PEPT2 (7.59 ± 0.98) pmol/mg protein relative to control (OAT2 9.49 ± 5.52; OAT3 2.79 ± 1.15; OAT5 8.35 ± 2.83; OATP2A1 0.45 ± 0.27; PEPT2 5.38 ± 0.98 pmol/mg protein). OCTN2 was the only uptake transporter decreased for MCD and Athero (4.80 ± 0.66 and 7.64 ± 2.19 pmol/mg protein, respectively) compared to control (10.9 ± 3.71 pmol/mg protein). However, CTR1 was not changed for MCD (0.52 ± 0.18 pmol/mg protein) but was the only transporter increased for Athero (0.88 ± 0.15 pmol/mg protein) relative to control (0.55 ± 0.27 pmol/mg protein). The only increases in uptake transporters for mice were in OAT3 for db/db (84.5 ± 26.5 pmol/mg protein) and in NTCP for FFDTH (3.70 ± 0.87 pmol/mg protein) relative to control (16.0 ± 2.30 and 2.54 ± 0.27 pmol/mg protein, respectively) (Figure 4).

Contrary to MCD, there was a general downward trend in transporter expression across the mouse models when examining significant changes. Decreases in expression for db/db, FFDTH, and ALIOS was found for OAT5 (2.27 ± 0.57, 7.97 ± 1.37, 14.1 ± 4.00); OCT3 (5.87 ± 0.23, 7.17 ± 0.64, 7.52 ± 1.97); OATP1A1 (0.29 ± 0.27, 0.83 ± 0.27, 1.72 ± 0.58); and OATP4C1 (1.76 ± 0.48, 1.33 ± 0.28, 1.71 ± 0.72 pmol/mg protein) relative to control (OAT5 23.0 ± 2.14; OCT3 9.83 ± 1.05; OATP1A1 3.06 ± 0.47; OATP4C1 3.33 ± 1.45 pmol/mg protein). Additionally, db/db and ALIOS also showed decreases in OATP3A1 (0.14 ± 0.11 and 0.14 ± 0.13 pmol/mg protein, respectively) and CTR1 (8.03 ± 1.32 and 9.48 ± 2.17 pmol/mg protein, respectively) relative to control (0.45 ± 0.28 and 14.0 ± 1.45 pmol/mg protein, respectively) and for FFDTH in OATP2A1 (0.78 ± 0.43 pmol/mg protein) relative to control (1.70 ± 1.07 pmol/mg protein). Along with the downward trend in uptake transporters for mouse models, SGLT2 is also decreased for FFDTH and ALIOS (1.45 ± 0.31 and 1.22 ± 0.12 pmol/mg protein, respectively) compared to control (2.05 ± 0.34 pmol/mg protein) (Figure 4).

**Protein Expression of Renal Efflux Transporters**

Significant changes in protein expression were also examined for renal efflux transporters. In keeping with the uptake transporters, MCD was skewed towards an upward trend with increases in MRP2 (1.50 ± 0.43; ENT2 20.1 ± 2.09; and MDR1b 7.22 ± 1.69 pmol/mg protein) relative to control (MRP2 0.83 ± 0.37; ENT2 14.9 ± 2.23; MDR1b 4.39 ± 1.70 pmol/mg protein). The only efflux transporter decreased for MCD was MRP3 (7.63 ± 1.47 pmol/mg protein) which is also decreased for Athero (8.40 ± 1.95 pmol/mg protein) relative to control (8.13 ± 1.10 pmol/mg protein). OCT3 was the only uptake transporter increased for Athero (0.88 ± 0.15 pmol/mg protein) relative to control (0.55 ± 0.27 pmol/mg protein). The only increases in efflux transporters for mice were in MRP2 for db/db (1.50 ± 0.43 pmol/mg protein) and in SGLT2 for FFDTH (1.45 ± 0.31 pmol/mg protein) relative to control (1.05 ± 0.28 pmol/mg protein) (Figure 4).
protein) compared to control (13.5 ± 4.94 pmol/mg protein). Additionally, Athero was decreased for MATE1 (29.1 ± 8.83 pmol/mg protein) compared to control (45.4 ± 19.7 pmol/mg protein) but was not changed for any of the other efflux transporters.

None of the efflux transporters demonstrated an increase for mouse models, apart from MDR1b for db/db (6.88 ± 0.36 pmol/mg protein) versus control (4.44 ± 0.51 pmol/mg protein) and MDR2 for db/db and FFDTH (7.05 ± 0.49 and 7.26 ± 1.03 pmol/mg protein, respectively) versus control (5.61 ± 1.34 pmol/mg protein). The only efflux transporter changed for all mouse models is the decrease in MRP4 for db/db, FFDTH, and ALIOS (1.72 ± 0.73, 1.44 ± 0.47, 0.91 ± 0.48 pmol/mg protein, respectively) relative to control (4.37 ± 0.83 pmol/mg protein). In addition to MRP4, db/db and ALIOS demonstrated a decrease in BCRP (0.69 ± 0.06 and 0.66 ± 0.28 pmol/mg protein, respectively) and in MRPl for ALIOS (1.88 ± 0.87 pmol/mg protein) compared to respective controls (BCRP 1.26 ± 0.34; MRPl 3.03 ± 1.16 pmol/mg protein) (Figure 5).

**Concordance Analysis of Rodent Models and Human NASH**

Effect sizes were estimated using Glass’s Δ to represent a concordance analysis (Equation 2) using quantified renal transporter protein expression for rodent models and previously reported human NASH data by Frost and Jilek et al. Effect sizes of the same direction (positive/positive or negative/negative) indicate protein expression changes in the same direction (decrease/decrease or increase/increase) (Frost and Jilek et al., 2022). On the contrary, effect sizes in opposite directions (negative/positive or positive/negative) indicate protein expression changes in contrasting directions (decrease/increase or increase/decrease). The magnitude represented by the distance a transporter is plotted from a respective axis, implies the statistical power to determine a change from control to NASH.

The uptake transporters with an upward trend in protein expression for human NASH are OCT3, OATP1A2/1A1, and PEPT2. The only similar trends were in OCT3 and PEPT2 for MCD and in OATP1A2/1A1 for Athero. BCRP is the only efflux transporter that was slightly increased in human that is also moderately increased for FFDTH, as well. The majority of uptake transporters (OAT1-4/5, OCT2, OATP4C1, OCTN1-2, and URAT1) and efflux transporters (MRP1-4, MATE1, ENT1-2, and MDR1) have a downward trend in human NASH. In the rodent models, OAT1 and OAT2 for ALIOS; OAT3 for FFDTH;
OAT 4/5 for db/db, FFDTH, and ALIOS; OCT2 for MCD, Athero, and ALIOS; OATP4C1 for Athero, db/db, FFDTH, and ALIOS; OCTN1 and OCTN2 for both MCD and Athero; and URAT1 for Athero, correspond to the decrease observed in uptake transporters for human NASH. The decreases that correspond to those observed for human efflux transporters are MRP1, 3, and 4 for all models; MRP2 for Athero and db/db; MATE1 for MCD, Athero, and db/db; ENT1 for MCD, Athero, and ALIOS; ENT2 for Athero, db/db, and ALIOS. Taken together, these observations suggest that the mouse FFDTH model has the closest directional trend comparisons (Figure 6).

**DISCUSSION**

Disease associated alterations in transporter expression can lead to pharmacokinetic variations in drugs administered at an approved FDA standardized dose that may result in toxicity or inadequate therapeutic efficacy (Evers et al., 2018). Hepatic transporter alterations are generally described in NASH as a decrease in basolateral uptake and canalicular efflux transporters and an increase in basolateral efflux transporters (Hardwick et al., 2011; Dzierlenga and Cherrington, 2018; Vildhede et al., 2020). These alterations can cause a shift in the distribution and elimination profile of various xenobiotics resulting in a higher systemic concentration, prolonged plasma retention, and decreased biliary excretion (Hardwick et al., 2012; Hardwick et al., 2014; Clarke et al., 2015; Toth et al., 2018; Toth et al., 2020). Similarly, alterations in renal transporters could result in pharmacokinetic variations. However, despite extensive clinical findings of renal dysfunction in NASH patients, renal transporter expression profiles in human NASH were unknown until recently (Targher et al., 2010; Musso et al., 2014; Droździk et al., 2020; Frost and Jilek et al., 2022) This characterization of renal transporter expression in human NASH allows the identification of a representative rodent model for future pharmacokinetic and toxicity studies, which can prove improve the clinical translatability of rodent studies investigating distribution and elimination variations of xenobiotics in NASH patients.

The present compilation of well-established rodent NASH models represents a variety of nutritional and genetic components to aid in the identification of the most appropriate model that best extrapolates human renal elimination alterations in NASH. The primary routes of renal elimination include
glomerular filtration and active tubular secretion through transporters in proximal tubule cells (Mathialagan et al., 2017). Therefore, GFR must be considered in each rodent model as well as the renal transporter expression profiles. While creatinine is a common and traditional endogenous biomarker used to determine estimated glomerular filtration rate, it has been criticized for the lack of sensitivity in detecting decreases in GFR and presents limitations in diagnosing impaired renal function. For improved sensitivity, it is recommended to use an exogenous biomarker for the determination of GFR (Delanaye et al., 2016). Therefore, iohexol clearance was used to evaluate GFR instead of the serum creatinine levels which were not significantly increased. In keeping with findings from other clinical studies, patients used for comparison in the current study exhibited a significant decrease in estimated GFR relative to control with each NASH patient having less than 50 mL/min/1.73m² (Targher et al., 2010; Frost et al., 2022). Although significant decreases in GFR were observed in the mouse models db/db, FFDTH, and ALIOS, in the rat models, only MCD showed a downward trend and Athero significantly increased relative to control. Therefore, it is important to determine if a xenobiotic undergoes glomerular filtration when designing a rodent study to accurately evaluate pharmacokinetics in NASH. In addition to GFR, substrate selectivity of transporters involved in active tubular secretion should be considered for a rodent pharmacokinetic study design as well.

Each rodent model investigated in this study demonstrated a variety of renal transporter expression changes, allowing future pharmacokinetic studies to consider the selectivity of each process to assist translation to clinical predictions. This can be exemplified using a previous study that investigated the pharmacokinetics of adefovir in the MCD NASH model (Laho et al., 2016). Adefovir is renally eliminated by basolateral uptake with OAT1 and OAT3 and apical efflux into the lumen with MRP4. However, contrary to the significant decrease observed for human NASH, OAT3 is significantly increased for the MCD model (Laho et al., 2016; Frost and Jilek et al., 2022) This weakens the clinical translatability for adefovir pharmacokinetics in NASH patients, which may be more accurately represented by a model that demonstrates a downward trend in OAT3, as seen with the FFDTH model. A significant decrease in apical transporter OAT4 was also observed in human NASH patients, but the impact of that change is complicated by the absence of a true OAT4 in rodents (Frost and Jilek et al., 2022). However, although not a true ortholog, OAT5 is expressed in rodents (but not humans) and shares some substrate...
and functional similarities with OAT4 (Jilek et al., 2021b). Therefore, the decrease in OAT5 expression observed in \(db/db\), FFDTH, and ALIOS should be considered for modeling human NASH pharmacokinetics of xenobiotics with substrate specificity to OAT4/5. In addition to considering the models that exemplify the significant decreases in OAT3 and OAT4 in human NASH, it is also important to consider substrate selectivity of transporters that are significantly changed in rodent models but not in human NASH. For instance, although MRP3 is not significantly changed in human NASH, it is significantly decreased in the rat models, MCD and Athero. As such, it would be important to consider the mouse models that are also not significantly changed for MRP3 when designing a pharmacokinetic study with an MRP3 substrate.

This survey of common rodent NASH models studied here offers a broad spectrum of physiological and renal transporter alterations which should prove useful in identifying the most appropriate model to accurately extrapolate pharmacokinetic or toxicokinetic changes in human NASH patients. Because no single rodent model exactly mirrors all transporter changes in human NASH, it is important to consider which specific transporter pathways are involved when modeling for each specific drug substrate. This investigation will enable improvements in clinical translatability of rodent NASH models to predict pharmacokinetic variations. Accurate extrapolation to human NASH would assist in developing future investigations of pharmacokinetic variations to predict dose adjustments that can prevent adverse drug reactions by achieving safe and effective systemic concentrations. These models represent a valuable resource for future investigations that will improve patient outcomes in this growing population.
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AUTHOR CONTRIBUTIONS

Participated in research design: Frost, Jilek, Toth, Wright, and Cherrington

Conducted experiments: Frost, Jilek, Toth, and Goedken

Contributed new reagents or analytical tools: Frost, Jilek, Toth, and Cherrington

Performed data analysis: Frost, Jilek, Goedken, Wright, and Cherrington

Wrote or contributed to the writing of the manuscript: Frost and Cherrington

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FOOTNOTES

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CONFLICT OF INTEREST: No author has an actual or perceived conflict of interest with the contents of this article.
FIGURE LEGENDS

Figure 1. Liver Pathology Scores. Mean ± standard deviation of overall liver injury scores assessed by a pathologist to confirm the development of NASH relative to respective controls in each model. All models, except for db/db, showed a significant increase in overall scores comprised of the hallmarks of NASH injury including, lipid accumulation, necrosis, inflammation, and fibrosis. Daggers or asterisks indicate significant statistical increase relative to rat control or mouse control, respectively (p ≤ 0.05).

Figure 2. Clinical Chemistry for Liver and Kidney Function. Concentrations of markers in plasma used to determine liver and kidney function represented as mean ± standard deviation. Alkaline phosphatase (ALP) significantly increased for MCD, db/db, and ALIOS models relative to respective controls. MCD and ALIOS significantly increased for alanine aminotransferase (ALT) relative to respective controls. All mouse models, including control, are elevated from the normal glucose range with db/db and FFDTH decreasing significantly relative to mouse control. FFDTH is the only model to significantly change in creatinine levels, however, MCD, Athero, and ALIOS models significantly increase relative to respective controls. GFR determined by percent change in iohexol clearance shows a significant increase in Athero but a significant decrease in db/db, FFDTH, and ALIOS relative to respective controls. Normal ranges are represented as dashed horizontal lines in grey for rats and blue for mice. Solid horizontal line separates rat from mouse. Statistical significance relative to respective rat or mouse controls are identified by daggers or asterisks for p ≤ 0.05, respectively.

Figure 3. Transcription Expression of Renal Transporters. Relative mRNA expression was determined using RT-qPCR and quantified with the 2−ΔΔCt method. A) Basolateral uptake transporters. Significant decrease in db/db for SLC22A6 (OAT1). Significant increase in SLC22A1 (OCT1) for MCD, Athero, and db/db relative to respective controls. Significant increase in SLC22A8 (OAT3) for db/db and decrease for FFDTH and ALIOS. Significant decreases for SLC22A2 (OCT2) in db/db and FFDTH. B) Apical uptake transporters. Significant decrease in SLC22A7 (OAT2) and SLCO1A1 (OATP1A1) for all mouse models relative to mouse control. C) Apical efflux transporters. No significant change for SLC29A1 (ENT1) in rat models relative to rat control. D) Apical efflux transporters. A significant decrease for ABCC2 (MRP2), ABCG2 (BCRP), and SLC47A1 (MATE1) for FFDTH compared to mouse control. Also, a significant
decrease for ABCC2 (MRP2) for ALIOS compared to mouse control. Solid horizontal line separates rat from mouse. Statistical significance (p < 0.05) represented as daggers for rats and asterisks for mice relative to rat or mouse control.

**Figure 4.** *Protein Expression of Renal Uptake Transporters.* Protein expression quantified by surrogate peptide LC-MS/MS in pmol/mg protein. A general upward trend for MCD with significant increases in OAT2, OAT3, OAT5, OATP2A1, and PEPT2 and the only decrease in OCTN2. Limited changes in uptake transporters for Athero with only CTR1 significantly increasing and OCTN2 decreasing. In contrast, a general downward trend was exemplified for the mouse models including significant decreases for db/db, FFDTH, and ALIOS in OAT5, OCT3, OATP1A1, and OATP4C1. Additional significant decreases were observed for db/db in OATP3A1, and CTR1, for FFDTH in OATP2A1 and SGLT2, and for ALIOS in OATP3A1, CTR1, and SGLT2. OAT3 in db/db and NTCP in FFDTH are the only significant increases for uptake transporters in the mouse models. Solid horizontal line separates rat from mouse. Statistical significance is defined as p < 0.05 relative to respective control represented by daggers for rats and asterisks for mice.

**Figure 5.** *Protein Expression of Renal Efflux Transporters.* Protein expression quantified by surrogate peptide LC-MS/MS in pmol/mg protein. MCD showed significant increases in MRP2, ENT2, and MDR1b and a significant decrease in MRP3. Athero only showed significant decreases which were in MRP1, MRP3, and MATE1. All mouse models, db/db, FFDTH, and ALIOS significantly decreased for MRP4. MRP1 significantly decreased for db/db and BCRP significantly decreased for db/db and ALIOS. FFDTH significantly increased for both MDR1b and MDR2 while FFDTH only significantly increased for MDR2. Solid horizontal line separates rat from mouse. Daggers for rats and asterisks for mice are used to identify statistical significance (p < 0.05) relative to respective controls.

**Figure 6.** *Concordance Analysis of Renal Transporters for Human and Rodent Models.* Effect sizes determined by Glass’s Δ for renal transporters are represented as rodent against human. Transporters moving in the same direction (positive/positive or negative/negative) demonstrate the same expression change while the distance from each axis represents the comparable magnitude of the change to detect a statistical difference between control and NASH. Transporters plotted in opposite directions...
(negative/positive and positive/negative) demonstrate contrasting trends in protein expression change for
rodent models compared to human NASH. OAT3, for example, is decreased in human NASH but was
increased in MCD, Athero, db/db, and ALIOS models placing it in the top left quadrant. However, OAT3
was decreased in FFDTH, placing it in the bottom left quadrant signifying a similar expression change
relative to human NASH.

Table 1. Surrogate Peptide Sequences and Transitions.

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Peptide sequences and respective transitions chosen for quantification, heavy labeled internal standard (IS), and retention times (RT) used for multiple reaction monitoring (MRM). Transitions used for quantification included doubly charged parent ion (Q1: [M+2H]^{2+}) and prominent singly charged fragment ion (Q3: [M+H]^+).