Transporter activity changes in non-alcoholic steatohepatitis: assessment with plasma coproporphyrin I and III

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MRP2/3/4, multidrug-resistance associated protein 2/3/4; OATP/Oatp, organic anion transporting polypeptide; CP I/III, coproporphyrin I/III; NASH, non-alcoholic steatohepatitis; LC-MS/MS, liquid chromatography–tandem mass spectrometry; ATP, adenosine triphosphate; AMP, adenosine mono-phosphate; ABC, ATP binding cassette; MEB, $^{99m}$Tc-mebrofrenin; MP, methapyrilene hydrochloride; CDAHFD, choline deficient amino acid defined high fat diet.

Abstract
Expression and functional changes in the organic anion transporting polypeptide (OATP)-multidrug resistance associated protein (MRP) axis of transporters are well reported in non-alcoholic steatohepatitis (NASH) patients. These changes can impact plasma and tissue disposition of endo- and exogenous compounds. The transporter alterations are often assessed by administration of a xenobiotic, or by transporter proteomic analysis from liver biopsies. Using gene expression, proteomics and endogenous biomarkers, we show that the gene expression and activity of OATP and MRP transporters are associated with disease progression and recovery in humans and in preclinical animal models of NASH. Decreased OATP and increased MRP3/4 gene expression in two steatosis and NASH patient cohorts, as well as gene and protein expression in multiple NASH rodent models have been established. Coproporphyrin I and III (CP I and III) were established as substrates of MRP4. CP I plasma concentration increased significantly in four animal models of NASH, indicating the transporter changes. Upto a 60-fold increase in CP I plasma concentration was observed in the mouse bile duct ligated model compared to sham controls. In the choline deficient high
fat diet (CDAHFD) model, CP I plasma concentrations increased by > 3-fold compared to chow diet fed mice. In contrast, CP III plasma concentrations remain unaltered in the CDAHFD model, although it increased in the other three NASH models. These results suggest that tracking CP I plasma concentrations can provide transporter modulation information at a functional level in NASH animal models and in patients.

**Significance Statement**

Our analysis demonstrates that MRP4 transporter gene expression tracks with NASH progression, and intervention in patients. Additionally, we show that CP I and III are substrates of MRP4. CP I plasma and liver concentrations increase in different diet and surgery-induced rodent NASH models, likely explained by both gene and protein level changes in transporters. CP I and III are therefore potential plasma based biomarkers that can track NASH progression in preclinical models and in humans.

**Introduction**

Non-alcoholic fatty liver disease (NAFLD) and non-alcoholic steatohepatitis (NASH) are global health concerns. NAFLD and progressive forms are commonly associated with lifestyle / dietary irregularities, high cholesterol, obesity, and type 2 diabetes. This spectrum of syndromes is characterized by accumulation of more than 5% fat in liver leading to hepatic steatosis (HS) (Chalasani et al., 2018). In a fraction of cases, often accompanied by inflammatory insults, HS can progress to NASH, a more severe form of liver disease with tissue fibrosis (Brunt et al., 2015).
It is reported that expression and function of drug transporters and drug metabolizing enzymes change during NASH. The hepatic uptake transporters (organic anion transporting polypeptide (OATP) 1B1, and OATP1B3) are downregulated, basolateral efflux transporters (multidrug-resistance associated protein (MRP)3, and MRP4) are upregulated and canalicular efflux transporter (MRP2) is mislocalized (Thakkar et al., 2017; Evers et al., 2018). Two mechanisms are associated with the expression alterations: (i) inflammatory cytokines released during progression of NASH (e.g. TNFα or IL-6) interact with nuclear receptors such as CAR, PXR, FXR or LXR (Cobbina, and Akhlaghi, 2017), or (ii) accumulated bile acids activate FXR (Lin and Kohli, 2018). Post-transcriptional modifications are associated with mislocalization and functional aberration of OATP and MRP2 transporter proteins during NASH (Clarke et al, 2017).

Plasma and hepatic concentrations of drugs that are substrates of OATP-MRP transporters are reported to increase due to these transporter alterations (Ali et al, 2018). Increased hepatic concentrations are reported for the diagnostic agent $^{99}$Tc-mebrofrenin (MEB) in NASH patients (Ali et al, 2018). Increased plasma concentrations are also reported for pravastatin, simvastatin acid, ezetimibe, and morphine glucuronides in different rodent models of NASH (Clarke et al, 2015; Clarke et al, 2014; Clarke et al, 2014; Dzierlenga et al, 2015; Hardwick et al, 2014; Laho et al, 2016; Lickteig et al, 2007; Suga et al, 2019). These pharmacokinetic alterations can potentially impact safety and efficacy of drugs in patient populations at different stages of disease. Hence, there is a need to understand the exposure changes in diverse NASH patient populations compared to healthy volunteers.

An endogenous compound that is a substrate of all the major hepatic drug transporters undergoing significant change in NASH (OATP1B1, OATP1B3, MRP2, MRP3, and MRP4), can provide functional level information about these transporters. Measuring plasma concentrations of an endogenous compound can avoid complications of linking mRNA or
protein levels to functional activity. An endogenous plasma based marker will therefore allow tracking of individual patients in terms of transporter function at particular NASH stages, and indicate disease progression.

Coproporphyrin I and III (CP I and III) are byproducts of heme biosynthesis. About 80% of CP biosynthesis takes place in erythrocytes (Wang et al, 2019). Elimination of CPs are completely transporter-mediated, primarily to urine and bile. They are known endogenous biomarkers for predicting OATP1B (SLCO1B) inhibition-based drug-drug-interactions (Lai et al, 2016, Shen et al, 2016). CP I concentrations have been recently used to monitor OATP1B activity in patients with end stage renal disease and recovery following kidney transplantation (Suzuki et al, 2019). In addition to OATP1B, we have shown CP I is a substrate of MRP2 (Gilibili et al, 2017). MRP2-mediated biliary clearance is compromised in Dubin Johnson syndrome (DJS). The increase in renal clearance of CP I and III suggests an involvement of hepatic basolateral efflux transporters to re-route clearance from biliary to renal side in DJS patients. A previous study indicated CP I and III as substrates of MRP3 (ABCC3), but not MRP4 (ABCC4) (Kunze et al, 2018). As CP I and III are known substrates for MRP2/3, and because MRP4 generally shares overlapping substrate specificity with MRP2/3, we re-evaluated the substrate potential of CP I and III towards MRP3 and 4 with a modified, highly sensitive method.

The aims of the current studies are to: (i) examine the changes in gene and protein expression of major hepatobiliary transporters in NASH patients and rodent models of NASH, and (ii) evaluate the effect of altered gene and protein expression of hepatobiliary transporters on plasma and liver concentrations of CP I and III, endogenous biomarkers of OATPs/MRPs in NASH animal models.
Materials and Methods

Chemicals and Reagents

CP I and CP III were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX). CP I-\(^{15}\)N\(_4\) sodium bisulfate salt from (Toronto Research Chemicals Inc., ON, Canada). Ethylacetate (AR grade, Spectrochem Pvt. Ltd, Mumbai, India). Human MRP3, and 4-expressing inside-out membrane vesicles (protein concentration 5 mg/ml) derived from Sf9 insect cells were purchased from GenoMembrane, Co., Ltd (Yokohama, Japan). Reaction incubation plates (96 well, ultra-low attachment, polystyrene, flat bottom, clear) purchased from Corning® Costar® (NY, USA). Assay plates (96 well, black polystyrene) for fluorescence measurement. All standard chemicals for vesicle assays were obtained from Sigma Aldrich (Bangalore, India).

Transcriptomic analysis of Canadian and French-Belgian cohorts

We utilized gene expression datasets with adequate clinical annotation from Canadian (GEO ID: GSE89632) and French-Belgian (GEO ID: GSE83452) cohorts of human NASH patients from Gene Expression Omnibus (GEO). Downloaded datasets were normalized in R Studio pipelines. Log2 normalized expression profiles of transporters (i.e. \(ABCC2\), \(ABCC4\), \(ABCC3\), \(SLCO1B1\), \(SLCO1B3\)) within the Canadian cohort were plotted as Healthy Control (Stage0:HC), Steatosis and NASH. The French-Belgian (FB) cohort was a longitudinal study with one year follow up for a subset of patients. Expression of these transporters was plotted for a subset of patients from the French-Belgian cohort who were initially classified NASH, with clinical resolution at one year following intervention with dietary restriction/bariatric surgery. The presence of NASH was defined according to NAS-CRN (clinical research network) guidelines, assessing combined presence of steatosis, ballooning and lobular inflammation (Chalasani et al., 2018).
Vesicular transport assay

The active uptake of CP I and CP III was assessed using inside out Sf9 insect cell derived vesicles over-expressing MRP3 or MRP4. The assay was conducted following methods standardized in-house for MRP2 vesicles (Gilibili et al, 2017). Briefly, the vesicle protein (0.2 mg/mL) was incubated for 20 min with either CP I (2 µM) or CP III (2 µM) in the presence or absence of inhibitors (100 µM MK-571 or 100 µM benzbromarone (BNZ)) followed by vacuum filtration. CP I and III were analysed using a fluorimeter (Perkin Elmer Envision). Concentration-dependent transport of CP I and III was assessed using optimized conditions for human MRP3, 4, and rat Mrp4 vesicles.

Animals

All animal experiments were performed in accordance with protocols approved by the respective Institutional Animal Ethical Committees. Male C57Bl/6 mice and male Sprague–Dawley (SD) rats were sourced as indicated. Animals were maintained in ventilated cages at 21 - 23°C with a 12 h light/dark cycle and food/water ad libitum. Experimental procedures were conducted in accordance with procedures set by the Committee for the Purpose of Control and Supervision on Experiments on Animals (CPCSEA), Government of India.

Bile duct ligation (BDL) model in mice and rats

Adult male C57Bl/6 mice (Envigo, Netherlands) 9-10 weeks old and Sprague Dawley rats (~8 weeks, 260–280 g body weight, Taconic, USA) were used in the studies. All animals were divided into bile duct ligated and sham control groups (n = 3 /group for rats, and n =6
mice in sham control group, and n=12 in mouse BDL group). Cholestatic liver injury was
induced by ligating the common bile duct in mice (Tag et al, 2015) and rats (Takita et al,
1988; Yang et al, 2015). To evaluate the time course of changes in expression of transporters
and CPs, blood and liver tissues were collected on day-0 (pre-surgery), 1, 3, 5, 7 and 14 days
post-surgery in rats. In mice, blood and liver tissue samples were collected on day 0 (before
surgery) and day 10. Blood (200 µL from rat and 100 µL from mice) was collected from each
animal, centrifuged at 10621x g for 3 min at 4˚C. The resultant plasma was separated and
stored in dark sample collection plates at -80 °C. A subset of 3 rats from both bile duct
ligated and sham control groups were euthanized on days-3, 7 and 14 after the plasma
collection. Rat and mouse livers were collected, weighed, and stored at -80 °C for further
analysis.

Rat methapyrilene-induced (MP) model of cholestatic liver injury

Male Sprague-Dawley rats (8-9 weeks, Taconic, USA) were administered methapyrilene
hydrochloride (MP) at a dose of 150 mg/kg body weight in phosphate buffer saline by oral
gavage 3 times per week as described (Probert et al., 2014). Rats were euthanized at 3 and 6
weeks of treatment followed by blood and liver collection for biochemical analyses and CP
quantitation.

Mouse NASH model: choline deficient amino acid defined high fat diet (CDAHFD)

Single housed C57BL/6J mice (6 weeks, Jackson Laboratories, Bar Harbor, ME) were
acclimated for 1 week on standard rodent chow (Teklad 2018, Madison, WI), followed by
specialized diets (Research Diets A06071302, New Brunswick, NJ). After 8, 18 or 22 (22
weeks with CDAHFD only) weeks on diet, mice (n=5 per time point) were sacrificed at time
points indicated from the control and CDAHFD diet groups, and blood samples were
collected. The blood samples were centrifuged at 4°C at 1500-2000 x g, plasma was removed, and stored at -80°C for later processing.

**Sample collection, RNA isolation and qPCR assay**

Rat, and mouse liver samples (BDL and methapyrilene studies) were collected in RNA Later (Invitrogen, Thermo-Fisher). Tissue lysis was done in Qiazol using Tissue lyser II (Qiagen). RNA was isolated through MN Nucleospin96 high throughput vacuum manifold and concentrations were established using a Thermo NanoDrop8000 Spectrophotometer. qPCR was performed for all samples in the panel of transporters depicted in Figure 3A and B. cDNA synthesis was done using 1000 ng of RNA as input from all samples using iScript™ RT Supermix as per manufacturer instructions. qPCR was set using SsoFast EvaGreen Supermix (BioRad) in 384 well format on a CFX384 instrument. For data analysis, ddCt with reference to housekeeping marker (*PPIA*) was calculated for each marker and fold change for BDL samples was normalized with respect to the sham control group.

**Membrane protein isolation and trypsin digestion procedure**

Liver (200 mg) from both sham and BDL rats were subjected to membrane fraction isolation procedure using the ProteoExtract Native Membrane Protein Extraction Kit from Calbiochem (CA, USA), as per manufacturer's protocol (Sun et al., 2005). Following trypsin digestion, peptides were subjected to mass spectrometric analysis. The procedures for membrane extraction, trypsin digestion and instrumentation are detailed in the supplementary information (S1). Selection of unique peptides was done as previously described (Gautam et al., 2018) and in S1.

**Instrumentation & chromatographic conditions for transporter protein quantitation**

Waters Acquity UPLC (ultra-performance liquid chromatography) Integrated System (Milford, USA) was used to inject 10 μL aliquots of the processed samples on a reverse-
phase column (Waters: BEH C18, 1.7 μm, 2.1 × 50 mm) maintained at 40 ± 2°C. The LC details can be obtained in S1. The analytical data were processed by Analyst software (v1.6.4). The optimized transitions are provided in Table 1.

The standard calibration curves using synthetic unlabelled peptides from New England Peptides (Boston, USA) were prepared in the range of 0.5 nM to 1 μM in bovine serum albumin (BSA, 0.1% in phosphate buffered saline). Sample preparation was as described for trypsin digestion procedure except for addition of labelled internal standards for each transporter protein. These peptides were added to 10% v/v formic acid and used during quenching of the tryptic digestion.

**LC-MS/MS Method for quantification of CP I and CP III**

LC-MS/MS based quantification of CP-I and CP-III from mouse and rat plasma and liver samples utilized a Acquity UHPLC (Waters Corporation, Milford, MA, USA) coupled with a AB Sciex 5500 Q-trap triple quadrupole mass spectrometer (AB Sciex, Framingham, MA, USA), fitted with an electrospray source in positive ionization mode. A labelled internal standard (Coproporphyrin I-15N4) was used for sample quantitation. The isomers of CP were baseline separated using an Ace Excel2 C18 column (150x2.1 mm) (Advanced Chromatography Technologies Ltd., Aberdeen, Scotland) and binary gradient elution comprising Mill-Q water with 0.1% formic acid (solvent-A) and acetonitrile with 0.1% formic acid (solvent-B). The following mass spectrometric conditions were used during the analysis: MRM transitions CP-I (655.5 → 596.3), CP-III (655.5 → 596.3) and d4-CP-I (659.3 → 600.3); capillary voltage, 5500 V; nebulizer and drying gas (high purity nitrogen) 50 psi; and drying gas temperature 550°C.

Twelve working solutions of CP I and CP III (mixture of CP I and III) were prepared in DMSO. The calibration standards (0.02 to 1000 nM) were then prepared by spiking
respective working solutions in 1% bovine serum albumin (surrogate matrix). An aliquot of 50 µL from each calibration standard and plasma study samples were placed into Eppendorf tubes, along with 20 µL of internal standard (\(^{15}\)N\(_4\)-CP-I) and 600 µL of ethyl acetate, vortexed for 2 min and centrifuged for 10 min set at 14000xg. A supernatant of 400 µL was separated from each tube and dried in a nitrogen evaporator set at 40°C until complete dryness. Then the tubes were reconstituted with 150 µL of methanol:water, 70:30 %, v/v. The samples were transferred to a 96 well plate and 4 µL injected on LC-MS/MS. Analyst version 1.6.2 was used for system control and data processing.

**Statistical Analyses**

Gene expression microarray datasets from both French Belgian and Canadian cohorts were normalized with RMA (Robust Multi-Array Average Expression Measure), a function from affy package (version 1.50.0) in R statistical software (version 4.0.2). Normalized datasets were then imported to Graphpad Prism to estimate statistical significance of transporters expression difference across three groups in Canadian cohort (Brown-Forsythe and Welch’s ANOVA tests with residual plots to account for independent variables). For all datasets with only 2 conditions, an unpaired t test (assuming Gaussian distribution) with Welch’s correction was performed. Contribution of different clinical covariates to gene expression of these transporters were calculated using variancePartition package (version 1.18.3) in R (version 4.0.2) which implement linear mixed model to calculate variance contributed by each clinical covariate.

**Results**

**Hepatobiliary transporter expression in NASH patients**
We evaluated the gene expression of key liver transporters in two distinct publicly available, clinically annotated cohorts of NASH patients. Global expression profiling in the Canadian cohort included healthy controls (n=24), steatotic (n=20) and NASH (n=19) livers in a cross-sectional study that included anthropomorphic, liver function, pathology and liver lipid profile data. MRP4 (ABCC4) showed a significant trend of increase compared to healthy control samples or steatotic liver biopsies (p<0.001 and p<0.01 respectively) (Figure 1A). In addition, upregulation of MRP3 and OATP1B1 expression was also observed (p<0.05 and p<0.08, respectively). We also mapped clinical covariates to the expression profiles of these genes (Figure 1B). These results suggest that MRP4 and possibly MRP3 and OATP1B1 are associated with NASH in humans.

**CP I and III are substrates of MRP4**

As a prelude to further evaluate MRP3 and MRP4 function, we assessed CP I and III as substrates of these transporters. Both CP I and CP III were found to be substrates of MRP3 and MRP4 under the conditions employed in these studies. Uptake of CP I and CP III in MRP3 and MRP4 vesicles was inhibited by the known inhibitors MK-571 and BNZ (Figure 2A and B). The ratio of uptake in the presence of ATP to uptake in the presence of AMP in MRP3 vesicles was 25.7 (CP I) and 25.1 (CP III), which decreased to 2.4 and 0.9 (for CP I) and 3 and 2 (for CP III) in the presence of MK-571 and BNZ, respectively. The ATP/AMP uptake ratio of CP I and CP III against MRP4 was 45.3 and 37.7, which decreased to 4.2 and 1.8 (for CP I) and 8.2 and 5.4 (for CP III) in the presence of MK-571 and BNZ, respectively. The affinities (Km) of transport by MRP3 and 4, for both CP I and CP III were found to be similar, 3-6 µM (supplementary table 1). Similarly, the Km of CP I in rat MRP4 is also similar to human MRP4, suggesting no significant interspecies difference in affinities.

**Hepatobiliary transporter expression in rodent NASH models**
We assessed the hepatobiliary transporter gene and protein expression changes in the rat and mouse BDL NASH models. A significant and gradual increase was observed in the $Abcc3$ gene from day-7 to day-14 in rat BDL livers (Figure 3A and 3B). For the solute linked carrier organic anion ($Slco$) transporters, such as $slco1a1$, $1a4$, $1b2$, $2b1$, and $1a5$ gene expression decreased with time. Similar changes in gene expression were observed for $Abcc3$, and the $Slco$ family of transporters in MP-treated rat livers (Supplementary file S2). Significant increase in $Abcc4$ was observed in mouse BDL livers compared to sham control. Interestingly, $Slco1a4$ increased by 4-fold, while $Slco1a1$ and $Slco1b2$ decreased significantly in BDL mice compared to sham control (S2).

An LC-MS/MS based proteomics approach was employed to assess changes in hepatobiliary transporter proteins in rat BDL livers (Table 1). Significant decreases in protein expression of Oatp1a1, 1a4, and 1a6 were observed in BDL livers compared to sham control rat livers on day-14 (Figure 3C-H). In contrast, Oatp1b2 protein expression did not change between sham control and rat BDL liver. Mrp3 protein could not be detected in rat livers. Trends to increased Mrp4 expression was observed on day-7 compared to sham control livers, while there was no change in Mrp2 protein expression (Figure 3C and 3D).

**CP I and III plasma and liver concentrations increase in the rat BDL model**

CP I plasma concentration increased 4.5-fold and CP III plasma concentration increased 7-fold on day-14, compared to day-0 plasma concentrations in sham rats (Figure 4A and B). As day-0 plasma concentrations provided the highest CP I and III concentrations in sham animals across different days, all comparisons are made with day-0 CP I and III plasma concentrations. The liver CP I and III concentrations (ng/g of liver) increased 1.4-2.8-fold in BDL rats compared to sham treated rats across different days (Figure 4C and D).
CP I and III plasma and liver concentrations also increase in mouse BDL model

A 40-60-fold increase in plasma concentrations of CP I and III was observed in BDL mice (Figure 5A and B) at day-10 post-surgery, compared to sham control. This observation was verified across four independent experiments (data not shown). The liver CP I and III concentrations (ng/g of liver) increased by 9, and 26-fold respectively, in BDL mice compared to sham treated mice on day-10 post surgery (Figure 5C and D).

CP I plasma concentrations increase in alternate models of NASH in rats and mice

In order to further establish CP I and III as endogenous substrates for hepatobiliary transporters in NASH, we evaluated CP I and III in additional preclinical NASH models in mice and rats. In a dietary model of liver disease in mice, where animals develop chronic disease over several weeks on a choline deficient high fat diet (CDAHFD model), the increase in plasma concentrations of CP I is moderate (up to 3 fold), while no increase in CP III plasma concentrations were observed (Figure 6A and B). The rat MP-induced model provides a path to a longer term, slower developing, chemically induced, and surgery independent fibrotic disease that does not involve bile duct ligation but mimics many of its histopathological characteristics. A 4 and 7.5-fold increase in plasma concentrations of CP I and III, were observed after 6 weeks of MP-treatment, compared to saline treated rats (Figure 6C and D).

Discussion

In the transporter mRNA analysis, we found ABCC4 (ABCC corresponds to MRP protein) to increase significantly in NASH and steatotic patients compared to healthy subjects. Interestingly, clinical staging of NASH was the single largest covariate with ABCC4 expression in the Canadian cohort (Figure 1B). Other covariates include NAS CRN score,
body mass index, and diagnosis of liver disease. Of the five transporters evaluated, \textit{ABCC4} shows the strongest correlation with these parameters. Increased Abcc4 mRNA is also reported in CDAHFD model in mice (Suga et al., 2019). The French-Belgian (FB) cohort compared NASH patients with healthy controls. A one-year follow-up was performed on a subset of patients (n=14) with interventional treatment to demonstrate clinical stage reversal of disease. In this cohort, \textit{ABCC4} mRNA expression decreased after 1 year of bariatric surgery and dietary restrictions, compared to the same subjects at T0, when they were clinically classified with NASH (Figure 7). This suggests that transporter expression may be concurrently regulated with disease progression. Therefore, specific endogenous substrates of these transporters could be used as plasma based biomarkers to track disease progression. There are very few markers that can complement non-invasive imaging or elastographic measurements. Thus, this novel strategy can be a significant value addition to the field.

The changes in mRNA expression of transporters in animal models are more pronounced than in humans, with \textit{Abcc3} increasing 10-15-fold in both rat BDL and MP-induced rat models of fibrosis. A statistically non-significant trend to increased \textit{Abcc4} was also observed. \textit{Slco} transporter gene expression decreased 3-4 fold in both animal models. Interestingly, mouse BDL livers showed significant increase in \textit{Abcc4} gene. Maximum increase of CP I and III plasma concentrations were also observed in mouse BDL compared to sham control, suggesting possible correlation of CP I and III plasma concentrations with \textit{Abcc4} gene (Mrp4 protein) expression.

Unlike gene expression, protein expression of Oatp1b2 remains constant in the BDL rat livers until day-14. Oatp1a1, 1a4, and 1a6 protein expressions decreased at day-14 in BDL rat livers (Figure 3C-H). Mrp3 protein could not be quantitated in the rat BDL model because of the low expression level of Mrp3 in rats. The protein expression level of Mrp3 is lowest in rats compared to human, monkey and dog (Wang et al., 2015). Therefore, the increased mRNA of
Abcc3 could not be corroborated with protein expression. The disconnect between gene and protein level changes reinforces the need for functional activity information of the OATP-MRP axis transporters.

CP I and III are endogenous compounds reported to be substrates of OATP1B, MRP2 and MRP3 transporters (Lai et al, 2016; Shen et al, 2016; Gilibili et al, 2017; Kunze et al., 2018). Plasma and urinary CP I and III have previously been utilized as biomarkers of OATP1B and MRP2 transporter function \textit{in vivo} (Lai et al., 2017; Wolkoff et al., 1976; Kondo et al., 1976; Benz-De et al., 2014). In order to complete the OATP-MRP axis of transporters, we evaluated CP I and III as substrates for MRP4. Additionally, the significant association of MRP4 with disease progression highlighted the importance of investigating any potential MRP4 probe substrate. A previous study (Kunze et al., 2018) did not find a sufficient signal for CP I and III against MRP4 in a vesicle assay. However, our studies demonstrate a significant signal to noise ratio for CP I and III against both MRP3 and 4. Differences in study design, e.g. detection method (fluorescence \textit{versus} radioactivity), vesicle source (HEK cells from PharmTox \textit{versus} Sf9 from Genomembrane), or final eluant (methanol \textit{versus} sodium dodecyl sulfate 0.5% in water) may have contributed to the differences between Kunze et al and our study. This is the first report showing CPI and III as substrates of MRP4. Therefore, plasma concentrations of CP I and III become potential candidates to understand NASH-related changes in the OATP-MRP axis of transporters.

The expression and functional changes in OATP-MRP axis of transporters, \textit{viz.} (i) decreased OATP-mediated uptake, (ii) increased MRP3/4-mediated basolateral efflux, or (iii) mislocalization of MRP2 can lead to increased plasma and liver concentrations of drugs and endogenous compounds. To evaluate if CP I and III follow the transporter changes observed in NASH, plasma and liver concentrations in various NASH rodent models were assessed. To our knowledge, this is the first report of plasma and liver concentrations of CP I and III.
compared across different preclinical models of NASH. There is no single animal model that completely mimics human NASH pathophysiology. Therefore, we assessed the plasma concentration of CP I and III in: (i) surgical NASH models, both rat and mouse BDL where cholestatic disease is observed (mouse BDL is accompanied by gall bladder dilation). Cholestatic liver injury is a major cause of liver fibrosis and cirrhosis in patients with either acute or chronic liver disease; (ii) MP-induced fibrosis in rat, a chemical-induced NASH to mimic bile duct ligation in a more chronic mode; and (iii) in a mouse diet-induced NASH model CDAHFD). This is a chronic model that factors in steatosis, a hallmark of human disease. However, the metabolic profile of this model does not completely reflect all properties of NASH in humans (reviewed by Liedtke et al., 2013 and Liu et al., 2013).

We have previously optimized all these models to demonstrate fibrosis at the time points studied, as evidenced by hydroxyproline levels and histopathological evaluation (e.g. both rat and mouse BDL models show portal and bridging fibrosis without cirrhosis at day-14 and day-10 respectively). In all models, significant increase in CP I plasma concentration was observed, whereas CP III plasma concentration increased in all but the CDAHFD model. Diet-induced models have been suggested to reflect human relevant transporter changes (Li et al., 2018). Hence, CP I may be more sensitive than CP III as a marker of transporter activity changes. Across different models and modes of NASH induction, the observed increase in CP plasma concentration suggests that they can be utilized as endogenous plasma based biomarkers, especially in conjunction with other small molecules e.g. bile acids (Puri et al., 2018; Luo et al., 2019).

The increase in liver concentrations of CP I and III are consistent with previous reports with MEB (Ali et al., 2018). Similar to rat BDL, in mouse BDL experiments, CP I and III hepatic concentrations also increased significantly (Figure 4 and 5). Although we have not evaluated Mrp2 internalization in this work, this phenomenon is heavily reported in NASH and
cholestasis models including rat BDL (Paulusma et al., 2000). Previous reports demonstrated a 60% reduction in biliary elimination in pemetrexed (Mrp2 substrate) in the diet-induced NASH model, without any statistically significant change in mRNA of protein expression (Dzierlenga et al., 2016). Mrp2 internalization will have a significant impact on hepatic concentrations of compounds. The hepatic concentration changes can impact efficacy of the compounds directly if the intended target is in liver. In addition, for compounds where the mechanism of toxicity is linked to liver targets (e.g. drug-induced cholestasis), increased hepatic concentrations can potentiate safety risks. Hence, changes in hepatic concentrations of drugs become critical owing possible effects on safety and efficacy. For understanding a direct role of MRP/Mrp2, 3, or 4 in plasma CP concentration changes during NASH, disease can be induced in individual transporter knock-out preclinical models. A complicating factor in the specificity of plasma CPs as NASH biomarkers could be the impact of NASH on the synthesis of CPs in erythrocytes and in liver. Although no reports were identified, the scope of this study cannot exclude such a complication. Future work using a labelled precursor of CP biosynthesis may address the effects of NASH on CP biosynthesis.

There are different approaches to qualitatively or quantitatively assess the impact of transporter alterations in NASH. Repeated liver biopsy on the same subject is challenging in a clinical setting. In addition, translating mRNA expression to functional activity can be difficult. Another potential method to assess transporter activity changes is to evaluate the pharmacokinetics of a probe compound (which is a substrate of the relevant transporters) in a patient population. Ali et al employed the nuclear imaging agent $^{99m}$Tc-MEB in NASH patients to quantitate compound disposition (Ali et al., 2018). However, MEB is not a sensitive substrate of MRP3, as basolateral efflux is stimulated rather than inhibited by the MRP3 inhibitor MK-571 (Swift et al., 2010). In another recent approach, a PBPK model based on transporter proteomics data from NASH and steatotic liver samples was utilized.
(Vildehede et al., 2019). However, the protein expression based PBPK model was unable to capture the liver accumulation of MEB. The model required a 5-fold decrease in MRP2 activity that could not be attributed to the protein expression changes alone. This highlights some key drawbacks of proteomics based PBPK to predict transporter activity changes. An endogenous compound with plasma concentrations that are sensitive to transporter functional changes can overcome the challenges to assess the impact of NASH on transporters – an important aspect as new therapies for the disease enter clinical trials. Tracking CP I/III plasma concentrations in patients can facilitate dose adjustment strategies for transporter substrates used or to be used in NASH treatments. To further evaluate CP I and III as potential plasma based biomarkers in NASH, the following experiments can be carried out: (i) evaluate the effect of drug treatment in preclinical models on plasma concentrations of CP I/III, (ii) evaluate the effect of disease and clinical stage reduction in human plasma with concentrations of CP I and III, and (iii) evaluate the association of CP I and III with other histological/imaging based markers of NASH.

In summary, liver transporters MRP4, MRP3, and OATP1B1 are altered in patients with clinically staged fatty liver disease and NASH. These changes are also observed in various preclinical models of NASH in rodents. In addition, we have shown that CP I and III are substrates of the transporters of the OATP-MRP axis. Altered expression of OATP-MRP transporters can also be correlated with the pharmacokinetic changes of CP I and CP III observed in preclinical NASH models. Taken together, these results suggest that liver transporters are modified in NASH patients, which may impact the disposition of endo- and xenobiotics. In addition, this study provides experimental evidence that CP I and III can be used as potential plasma based biomarkers of NASH progression.
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Wrote Manuscript: SC, SM, MS, HS
Designed Research: SC, SM, HS
Performed Research: SSLVJ, TN, SSG, BVM, AAH, VN, SB, GG, BAZ, YZ
Analyzed Data: SSLVJ, TN, AD, BVM, VK, BAZ, TTM, SC, SM, HS
Contributed New Reagents/Analytical Tools: TTM, SM, SC, HS, MR, MS

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Footnotes
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Figure 1A-B Global gene expression profile analysis of Canadian NAFLD/NASH cohorts. The Canadian cohort includes clinically staged samples encompassing healthy controls, NAFLD and NASH. In Figure 1A and B, 200 ng quality checked (Agilent BioAnalyzer) RNA from these annotated samples were labelled and amplified following Illumina Whole Genome Expression - DASL assay kit and subject to global expression profiling as indicated with GEO ID GSE89632. In Figure 1B, annotated clinical covariates were compared to transporter expression levels. Further analysis and statistical evaluation was performed as described in Methods. ***: p < 0.001, *: p < 0.05.

Figure 2: CP I (Figure 2A) and III (Figure 2B) transport in MRP3 and 4 overexpressed membrane vesicles in the presence and absence of inhibitors (MK-571 and benzbromarone). Fluorescence intensity as an average from 3-wells were plotted for each conditions. ***: p<0.001, **: p < 0.01.

Figure 3: Transcriptomic and proteomic analysis of hepatic transporters in rat BDL model of fibrosis. Figure 3A-B: qPCR based mRNA estimation of Abcc2, Abcc3, Abcc4, slco1a1, 1a4, 1b2, 2b1 and 1a5 genes from day-7 and day-14 liver samples (n=4) obtained from the rat BDL study. *: p < 0.05, **: p < 0.01, ***: p < 0.001. Figure 3C-H: Proteomics analysis of hepatic transporters in rat BDL study. LC-MS/MS-based protein quantification of day-7 and day-14 liver samples from the rat BDL study conducted for Mrp2,4, Oatp1a1,1a4, 1a6, and 1b2 proteins. *: p < 0.05, **: p < 0.01, ***: p < 0.001.

Figure 4A-D: CP I and III plasma concentration (A and B) and liver accumulation (C and D) in bile duct ligated SD rats. Bile duct ligation was conducted on day-1, while samples were taken on day-0 (pre-surgery, n=12), day-1 (n=12), day-3 (n=12), day-5 (n=8), day-7 (n=8), day-10 (n=4) and day-14 (n=4). Plasma concentrations from BDL rats were compared with
day-0 sham control rats (n=4), while liver accumulation compared with corresponding sham control liver. *: p < 0.05, **: p < 0.01, ***: p < 0.001.

**Figure 5A-D:** CP I and III plasma concentration (A and B) and liver accumulation (C and D) in bile duct ligated C57BL/6 mice. Bile duct ligation was conducted on day-3, while samples were taken on day-10, at the end of the study. Plasma concentrations from BDL mice (n=12) were compared with day-10 sham control rats (n=6), while liver accumulation in BDL mice were compared with corresponding sham control liver. **: p < 0.01, ***: p < 0.001.

**Figure 6:** CP I and CP III concentrations in other preclinical models across species. **Figure 6A-B:** CP I and III plasma concentration in choline-deficient high fat diet fed mice. At 7 weeks of age mice were placed on either a matched normal chow diet containing standard levels of choline, methionine and fat (Research Diets A13012807, New Brunswick, NJ) or were placed on a choline-deficient amino acid defined (methionine- 0.1%), high fat (60% of calories from fat) diet, CDAHFD. Plasma samples collected from each animal (n=5 at each time point) at 8, 18 or 22 weeks. **Figure 6C-D:** CP I and III plasma concentration in methapyrilene-induced SD rat model of NASH. Plasma CP concentrations from PBS dosed rats (n=5, each of 3 and 6 weeks), were compared with plasma concentrations from 150 mg/kg methapyrilene treated rats (po, thrice weekly). Number of rats from treatment group are 5 and 4, for 3rd and 6th week of treatment respectively. *: p < 0.05, **: p < 0.01, ***: p < 0.001.

**Figure 7:** Transporter gene expression in patients with clinical stage reduction in disease. The French-Belgian cohort established gene expression profiles in a large cross-sectional study. The subset depicted herein tracked 14 NASH patients post-1 year of interventional therapy (post-tx), with clinical stage reduction in disease. The FB cohort was profiled for
global gene expression using GeneChip Human Gene 2.0 ST Array, as documented with GEO ID GSE83452.
Table: 1  
Optimized mass transitions for the labelled and unlabelled peptides for the rat liver transporter quantitation.

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Note: The peptides represented in italic are the labelled peptides.
Figure 3

Fig 3A

Day 7

Sham control
BDL

Normalized fold expression change

Abcc2  Abcc3  Abcc4  Slco1a1  Slco1a4  Slco1b2  Slco2b1  Slco1a5

Fig 3B

Day 14

Sham control
BDL

Normalized fold expression change

Abcc2  Abcc3  Abcc4  Slco1a1  Slco1a4  Slco1b2  Slco2b1  Slco1a5

Figure 3C: Mrp2

Fig 3D: Mrp4

Fig 3E: Oatp1a1

Time post-surgery (days)

Sham control
BDL

fmol/μg of Abcc2 membrane protein

3 7 14

Sham control
BDL

fmol/μg of Abcc4 membrane protein

3 7 14

Sham control
BDL

fmol/μg of Slco1a1 membrane protein

3 7 14

Figure 3F: Oatp1a6

Figure 3G: Oatp1a4

Figure 3H: Oatp1b2

Time post-surgery (days)

Sham control
BDL

fmol/μg of Slco1a6 membrane protein

3 7 14

Sham control
BDL

fmol/μg of Slco1a4 membrane protein

3 7 14

Sham control
BDL

fmol/μg of Slco1b2 membrane protein

3 7 14
Figure 4

Figure 4A

CP I concentration (nM)

Time (days)

0 1 3 5 7 10 14

Sham control
BDL

***

Figure 4B

CP III concentration (nM)

Time (days)

0 1 3 5 7 10 14

Sham control
BDL

**

*

Figure 4C

CP I amount (ng/g)

Time (days)

3 7 14

Sham control
BDL

**

Figure 4D

CP III amount (ng/g)

Time (days)

3 7 14

Sham control
BDL