

**Interindividual variability in hepatic OATPs and P-glycoprotein (ABCB1) protein expression: Quantification by LC-MS/MS and influence of genotype, age and sex**

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**Running Title:** Interindividual variability in hepatic transporter expression

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**ABBREVIATIONS:** Area under the curve, AUC; Breast cancer resistance protein, BCRP; Clearance, CL; *In vitro* to *in vivo* extrapolation, IVIVE; liquid chromatography tandem mass spectrometry, LC-MS/MS; ; multidrug resistance associated protein 2, MRP2; Multiple reaction monitoring, MRM; organic anion-transporting polypeptide (OATP); P-glycoprotein, P-gp; quality control, QC; physiologically based pharmacokinetic, PBPK; pharmacokinetic-pharmacodynamic, PKPD; single nucleotide polymorphism, SNP; stable isotope label, SIL

## ABSTRACT

Interindividual variability in protein expression of organic anion-transporting polypeptides (OATPs), OATP1B1, OATP1B3, OATP2B1, and multidrug resistance -linked P-glycoprotein (P-gp) or ABCB1 was quantified in frozen human livers (n=64) and cryopreserved human hepatocytes (n=12) by a validated LC-MS/MS method. Membrane isolation, sample workup and LC-MS/MS analyses were as described before by our laboratory. Briefly, total native membrane proteins, isolated from the liver tissue and cryopreserved hepatocytes, were trypsin digested and quantified by LC-MS/MS using signature peptide(s) unique to each transporter. The mean  $\pm$  SD (maximum/minimum range in parentheses) protein expression (fmol/ $\mu$ g of membrane protein) in human liver tissue was, OATP1B1:  $2.0 \pm 0.9$  (7), OATP1B3:  $1.1 \pm 0.5$  (8), OATP2B1:  $1.7 \pm 0.6$  (5), and P-gp:  $0.4 \pm 0.2$  (8). Transporter expression in the liver tissue was comparable to that in the cryopreserved hepatocytes. Most importantly, livers with *SLCO1B1* (encoding OATP1B1) haplotypes \*14/\*14 and \*14/\*1a (i.e., representing SNPs, c.388A>G, and c.463C>A), had significantly higher ( $P < 0.0001$ ) protein expression than the reference haplotype (\*1a/\*1a). Based on these genotype-dependent protein expression data, we predicted (using Simcyp) up to ~40% decrease in mean area under the curve (AUC) of rosuvastatin or repaglinide in those individuals harboring these variant alleles compared with those harboring the wild-type alleles. *SLCO1B3* (encoding OATP1B3) SNPs did not significantly affect protein expression. Age and sex were not associated with transporter protein expression. These data will facilitate prediction of population-based human transporter-mediated drug disposition, drug-drug interactions, and interindividual variability through PBPK modeling.

## INTRODUCTION

Hepatic transporters, present at the sinusoidal or canalicular membrane, can determine plasma concentration of drugs by affecting their metabolic and/or biliary clearance (Backman et al., 2002; Schneck et al., 2004; Shitara et al., 2004; Shitara et al., 2006; Giacomini et al., 2010; Schipani et al., 2012). Consequently, these transporters can affect the efficacy (Bailey et al., 2010; Tomlinson et al., 2010) and/or toxicity (Alexandridis et al., 2000; Bosch Rovira et al., 2001; Marsa Carretero et al., 2002) of drugs by modulating their exposure to the target sites (Harwood et al., 2013). Hence, it is important to delineate the role of hepatic transporters in drug disposition and local tissue drug exposure, particularly because plasma drug concentrations are generally used as a surrogate measure of tissue concentrations to describe pharmacokinetic-pharmacodynamic (PK/PD) relationships and to predict drug-drug interactions (DDIs) or drug-gene interactions (Lon et al., 2012; Harwood et al., 2013). To achieve these goals on a population basis, physiologically based pharmacokinetic (PBPK) models (e.g., Simcyp) are increasingly being used in drug development and pharmaceutical research (Varma et al., 2012; Varma et al., 2013). For drugs where transporters are involved in their disposition, successful use of PBPK models requires critical information on the tissue localization and expression of the transporters including the effect of covariates, like, genotype, age and sex on transporter expression (Deo et al., 2012; Chu et al., 2013; Harwood et al., 2013; Prasad et al., 2013). However, such data are currently not available (Harwood et al., 2013). Here we report protein quantification data on the hepatic transporters as a start to fill this crucial knowledge gap.

Recent US-FDA draft guidance on pharmacokinetic DDIs

(<http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/ucm292362.pdf>) has highlighted the clinical importance of hepatic organic anion-transporting polypeptide transporters (OATPs), ABC drug transporter ABCB1 or P-glycoprotein (P-gp) and breast cancer resistant protein (BCRP or ABCG2) due to their broad substrate specificity and the potential to be involved in DDIs. We (Deo et al., 2012; Prasad

et al., 2013) and others (Balogh et al., 2012; Bi et al., 2012; Kimoto et al., 2012; Ohtsuki et al., 2012; Schaefer et al., 2012; Tucker et al., 2012) have reported data on expression of some of these hepatic transporters. Here we have extended these studies to determine 1) the inter-individual variability in expression of OATP1B1 (*SLCO1B1*), OATP1B3 (*SLCO1B3*), OATP2B1 (*SLCO2B1*) and P-gp (*ABCB1*) in a large set (n=64) of human liver samples; and 2) the influence of genotype, age and sex on such expression.

## MATERIALS AND METHODS

### Chemicals and reagents

The ProteoExtract native membrane protein extraction kit was procured from Calbiochem (Temecula, CA). The protein quantification BCA kit and the in-solution trypsin digestion kit were purchased from Pierce Biotechnology (Rockford, IL). Synthetic signature peptides (Table 1) for OATP1B1, OATP1B3, OATP2B1 and P-gp were obtained from New England Peptides (Boston, MA). The corresponding stable isotope labeled (SIL) internal standards, were obtained from Thermo Fisher Scientific (Rockford, IL). HPLC-grade acetonitrile was purchased from Fischer Scientific (Fair Lawn, NJ), and formic acid was purchased from Sigma-Aldrich (St. Louis, MO). All reagents were analytical grade.

### Human liver samples and hepatocytes

Sixty four liver tissue samples from the human liver bank of the School of Pharmacy, University of Washington, were used. Most of the subjects were Caucasians (except one Asian male [HL165] and three Black males not of Hispanic origin [HL104, 105 and 137]); the subject ages ranged from 7-70 yr with 30 females and 34 males. Procurement (approved by the UW Human Subjects Division), characteristics and storage of these samples has been previously described (Paine et al., 1997) and additional details are provided in Supplemental Table 1. The majority of these livers were obtained from organ donors who met with accidental death (e.g. trauma from vehicular accidents, subarachnoid hemorrhage, cerebrovascular accident) and were harvested from breathing donors who were perfused with UW solution for organ transplant purposes. The majority of subjects were not on any chronic medication, but did receive medication in the intensive care unit prior to tissue harvest. Cryopreserved human hepatocytes (7 batches of individual and 5 batches of pooled hepatocytes, Table 2) were procured commercially from Celsis IVT (London, UK), Xenotech (Lenexa, KS), Life Technologies (Carlsbad, CA) and BD Gentest (San Jose, CA).

### **Membrane protein extraction and total protein quantification**

The liver tissue (~100 mg) was processed, as we have described before, to isolate total membrane proteins (Calbiochem, Temecula, CA) (Deo et al., 2012; Prasad et al., 2013). Briefly, the tissue was homogenized in 2 ml extraction buffer I (ProteoExtract™ native membrane protein extraction kit) containing protease inhibitor cocktail (10 µl) of the kit and incubated with gentle shaking for 10 min. The resultant homogenate was centrifuged at 16,000xg for 15 min and the supernatant was discarded. The pellet was resuspended in 1 ml extraction buffer II of the kit with 10 µl of protease inhibitor cocktail. The latter was incubated with gentle shaking for 30 min at 4°C followed by centrifugation at 16,000xg for 15 min at 4°C. Total isolated membrane protein concentration (i.e., the supernatant) was determined using the BCA protein assay kit. The supernatant was diluted to a working concentration of 2 µg protein/µl or lower. Similar to the tissues, the pellet of cryopreserved hepatocytes (2-5 x 10<sup>6</sup> cells) were processed as discussed above except that the cells were washed twice with washing solution of the kit before addition of 2 ml extraction buffer I. The remaining procedure was the same as described for the liver tissue.

### **Purification of human Pgp**

Human Pgp from crude membranes of High Five insect cells was purified as described previously (Sauna et al., 2006) with some modifications. Briefly, the crude membranes were solubilized with n-dodecyl-β-D-maltoside (DDM) (1.00% w/v) in the presence of 20% glycerol. Solubilized proteins were subjected to metal affinity chromatography (Ni-NTA resin; Qiagen Inc, Valencia, CA) in the presence of 0.51 mM DDM; purified Pgp was eluted with 200 mM imidazole. The eluate was further purified by gel filtration chromatography using superdex S-200 column to remove imidazole. The fractions containing P-gp were then concentrated using Amicon ultrafiltration concentrators with 100 KDa cutoff (EMD Millipore, Billerica, MA) to ~0.5-1.5 mg/ml and stored at -70 °C. The protein concentration was quantified using the BCA protein assay kit as per manufacturer's instructions.

## Peptide selection, trypsin digestion of membrane proteins and sample preparation

Two unique signature peptides (not present in any other known protein), were selected for quantification of each transporter (Table 1) based on previously reported criteria (Kamiie et al., 2008) and literature reports (Zhang et al., 2011; Balogh et al., 2012). Briefly, peptides with transmembrane regions, single nucleotide polymorphisms (SNPs), posttranslational modifications or those susceptible to degradation were not selected. Continuous R and K sequences (RR, RK, KR and KK) were excluded to avoid the miscleavages by trypsin. The length of selected peptides was between 9 to 16 amino acid residues. Selected signature peptides were NVTGFFQSFK/ YVEQQYGQPSSK (OATP1B1), NVTGFFQSLK/ IYNSVFFGR (OATP1B3), VLAVTDSPAR/ SSPAVEQQLLVSGPGK (OATP2B1) and NTTGALTTR/ IATEAIENFR (P-gp). The corresponding peptides containing labeled [ $^{13}\text{C}_6$   $^{15}\text{N}_2$ ]-lysine and [ $^{13}\text{C}_6$   $^{15}\text{N}_4$ ]-arginine residues were used as the internal standards.

Trypsin digestion conditions were optimized for time (24 h) and protein:trypsin ratio (25:1; w/w). 20  $\mu\text{l}$  of 2.0  $\mu\text{g}/\mu\text{l}$  (or lower concentration) of tissue membrane preparation were incubated with 4  $\mu\text{l}$  dithiothreitol (100 mM) and 4  $\mu\text{l}$  iodoacetamide (200 mM) in 10  $\mu\text{l}$  ammonium bicarbonate digestion buffer (50 mM, pH 7.8). The protein samples were digested by trypsin in a final volume of 60  $\mu\text{l}$  at 37 °C for 24 h and the reaction was quenched by 20  $\mu\text{l}$  of SIL peptide internal standard cocktail (prepared in 70% acetonitrile in water containing 0.1% formic acid) and 10  $\mu\text{l}$  of the neat quenching solvent. The samples were centrifuged at 4000 xg for 5 min. For calibration standards, the working solution (10  $\mu\text{l}$ ) of the standard cocktail was added in the last step instead of the neat quenching solvent.

## Analytical method parameters

Agilent 6460A triple-quadrupole mass spectrometer coupled to Agilent 1290 Infinity LC system (Agilent Technologies, Santa Clara, CA) operated in ESI positive ionization mode was used for LC-MS/MS analysis of the signature peptides. Approximately 2  $\mu\text{g}$  or lower of the trypsin digest (5  $\mu\text{l}$ ) was injected onto the column (Kinetex™ 2.6  $\mu\text{m}$ , C18, 100 x 3 mm,

Phenomenex, Torrance, CA) and eluted at 0.4 ml/min. The mobile phase gradient conditions were, 97% A (water containing 0.1% v/v formic acid) and 3% B (acetonitrile containing 0.1% v/v formic acid) held for 4 min, followed by seven steps of linear gradient of mobile phase B concentration of 3% to 12.5%, 12.5% to 18%, 18% to 19.5%, 19.5% to 20%, 20% to 35%, 35% to 50% and 50% to 90% over 4-8 min, 8-11 min, 11-13.5 min, 13.5-16 min, 16-18 min, 18-18.4 min and 18.4-18.6 min. This was followed by the washing step using 90% mobile phase B for 1.6 min, and re-equilibration for 4.8 min. The doubly charged parent to singly charged product transitions for the analyte peptides and their respective SIL peptides were monitored using optimized LC-MS/MS parameters (Table 1).

The calibration curve standards were prepared by spiking peptide standards into the extraction buffer II of the membrane protein extraction kit. Seven calibration concentrations; ranging from ~0.2 to 20.0 fmol (on column) were used. The analytical method was validated for accuracy, inter- and intra-day precision and stability (freeze and thaw, bench-top and autosampler conditions) as we have described previously (Prasad et al., 2013). The quality control samples prepared by spiking extraction buffer II or pooled human liver membrane were quantified after every 12-15 samples. In addition, reliability of surrogate peptide based LC-MS/MS protein quantification was validated using the only purified transporter available to us, namely P-gp. Four different concentrations of P-gp protein standard (2.2, 4.4, 17.7 and 35.3 fmol, on-column) were prepared as quality control samples and were then, processed as were the membrane extracts of the liver tissues. The data were processed by integrating the peak areas generated from the reconstructed ion chromatograms for the analyte peptides and their respective internal standards using the MassHunter software (Agilent Technologies, Santa Clara, CA). For quantification of samples or standards, peak response from the two transitions from each peptide was averaged.

### **Genotyping and genotype dependent changes on protein expression**

Genotyping data were kindly provided by Dr Yvonne Lin, Department of Pharmaceutics, University of Washington. Briefly, genomic DNA was extracted from liver tissues. The genotype analysis was done using Affymetrix DMET Plus Array (Santa Clara, CA) according to the manufacturer's protocol. Individual genotypes were resolved and the minor allele frequencies were determined using the Affymetrix DMET Plus console (version 1.1) using the Dynamic Genotype Boundaries algorithm. Unpaired t-test was used to compare protein expression observed in two genotypic groups. When comparing protein expression in three or more groups, the Bonferroni multiple comparison correction was applied.

### **PBPK simulations**

Effect of *SLCO1B1* polymorphism on the pharmacokinetics of OATP1B1 substrates, rosuvastatin and repaglinide was predicted using the population-based ADME simulator of Simcyp (version 12.0, SimCYP Ltd, Sheffield, UK). The rosuvastatin and repaglinide parameters of Simcyp library file were used as such except that the REF (Relative Expression Factor; Supplemental Table 2) of OATP1B1 was varied for a given genotype/haplotype as compared to the default Simcyp value for wild-type (set at 1). The 90% confidence intervals (CIs) of mean REFs were used to estimate population variability in each group (Supplemental Table 2). Expression of other transporters, i.e., OATP1B3, OATP2B1, BCRP and sodium-taurocholate cotransporting polypeptide (NTCP), was assumed constant for all simulations. Data for sixteen subjects (70 kg; age 18-65 years, proportion of females: 0.5), given a single dose of rosuvastatin (20 mg) or repaglinide (0.50 mg), were simulated (4 trials x 4 subjects).

## RESULTS

### Analytical methods

The calibration curves were linear across the calibration range ~0.2-20.0 fmol (on column). The lower limit of quantification (LLOQ) was between 0.1 to 0.3 fmol (on-column) for all the peptides (except YVEQQYGPSSK which had a LLOQ of 1.2 fmol). Accuracy and precision in the quantification of the QC samples were acceptable (CV<25%) at three different concentrations. Trypsin digestion of the transporters was maximized by monitoring the time at which there was no further increment in the yield of the peptide (data not shown). All the peptides were found to be stable during sample preparation (i.e., when exposed to three cycles of freeze and thaw, at bench-top for 6 h and in auto-sampler for 48 h).

Using the purified P-gp, our peptide based LC-MS/MS approach was able to recover the concentration of P-gp in the quality control samples with an accuracy of  $124.0 \pm 11.2\%$  and  $74.7 \pm 4.1\%$  using the P-gp surrogate peptides 1 and 2 (Table 1) respectively. For the remaining transporters, since purified protein standards are not available, the analytical method was based on the assumption that the proteins are completely digested by trypsin to their corresponding peptides and there is a complete extraction of membrane proteins from the tissue homogenate. Although we used two peptides to quantify each protein, only one peptide for OATP1B1, peptide 1, was detectable with acceptable sensitivity. For the remaining transporters, we observed a minor (up to 1.5 fold) but systematic difference ( $P < 0.05$ , paired t-test, Table 3) in protein quantification between the two different signature peptides. We assume that this difference was due to different degree of trypsin digestion. Therefore, the results reported here are based on the peptides that yielded higher protein expression, namely NVTGFFQSFK (OATP1B1), NVTGFFQSLK (OATP1B3), VLAVTDSPAR (OATP2B1) and NTTGALTTR (P-gp). Nevertheless, our final conclusions of the effect of genotype, age and sex on transporter expression were the same irrespective of the peptide used (see below).

### **Interindividual variability in transporter expression in human livers and cryopreserved human hepatocytes**

Our yield of total membrane protein was  $3.7 \pm 1.1$  mg/100 mg tissue. The expression of OATPs and P-gp in tissue samples was comparable to that observed in the cryopreserved hepatocytes (Table 3). Similar to human liver tissue, the expression of these transporters in hepatocytes were in order of OATP1B1 > OATP2B1 > OATP1B3 > P-gp. Interindividual variability, calculated as fold range in transporter expression, i.e., maximum/minimum expression, was 5-8 fold; see Fig 1. Expression of all the transporters was independent of age (Fig. 2) or sex (Fig. 3).

### **Effect of genotype on hepatic transporter protein expression**

Reported SNPs of all transporters (except *SLCO2B1*) were observed in our liver bank (Table 4). Amongst the OATPs, only OATP1B1 expression was genotype dependent. When analyzed individually (without regard to haplotypes), livers homozygous for the non-synonymous *SLCO1B1* SNPs, c.388A>G or c.463C>A, or the synonymous SNP, c.597C>T, expressed 1.8, 2.1 and 1.7-fold higher OATP1B1 protein than those with the corresponding wild-type allele, respectively ( $P < 0.0001$ ) (Fig. 4). Moreover, livers that were heterozygous for c.463CA had higher OATP1B1 expression than the wild-type livers ( $P < 0.0001$ ), and the expression in the former was comparable to that in homozygous livers, c.463AA (Fig. 4). The expression of OATP1B1 in livers carrying a single c.388AG, c.597CT, c.571TT, c.571TC or -1187GA allele was not significantly different from the wild-type livers. Only one liver in our tissue bank was homozygous for the functionally relevant SNP c.521T>C. OATP1B1 expression in livers with c.521TT vs. 521TC was not significantly different (Fig 4).

In agreement with other studies (Kalliokoski et al., 2008a; Nies et al., 2013), we observed significant linkage disequilibrium amongst the above SNPs. Therefore, we examined the

expression of OATP1B1 based on *SLCO1B1* haplotypes of the three previously described key variants (c.388A>G, c.463C>A and 521T>C; Fig. 5A). Hepatic OATP1B1 protein expression was significantly higher ( $P<0.0001$ ) in livers with *SLCO1B1* haplotypes, \*14/\*1a and \*14/\*14 as compared to those harboring the reference allele (i.e., \*1a/\*1a), \*15/\*1a or \*1b/\*1a (Fig. 5B). The carriers of haplotypes, \*14/\*1a and \*14/\*14 had respectively 1.9 and 2.2-fold higher OATP1B1 expression than those carrying the reference allele. Furthermore, livers homozygous for c.388A>G independently increased OATP1B1 protein expression even when carriers of both 463AC and 463AA were excluded (Fig. 5C). SNPs c.388A>G and c.521T>C show opposite effect on the pharmacokinetics of a number of drugs (Niemi et al., 2004; Kameyama et al., 2005; Hartkoorn et al., 2010; Rodrigues et al., 2011; Schipani et al., 2012; Sortica et al., 2012; Nies et al., 2013). In agreement to these observations, when homozygous alleles of 388A>G were excluded from analysis, we observed a modest but statistically significant ( $P<0.05$ ) decrease in OATP1B1 expression in individuals with c.521TC vs. c.521TT (Fig. 5C). An independent effect of SNP c.521CC (\*5) could not be examined because of its low frequency in our liver bank.

Subjects harboring *SLCO1B3* haplotype (c.334G>T, c.699A>G, c.1557G>A and c.1833A>G) did not affect protein expression. Genotype-dependent protein expression could not be investigated for P-gp due to the large number of haplotypes, each with a limited sample size. Individually, none of the *ABCB1* SNPs significantly affected protein expression (Table 4).

### **Quantitative impact of OATP1B1 polymorphism on pharmacokinetics of rosuvastatin and repaglinide**

Utilizing the protein expression data (Figs. 4, 5 and Supplemental Table 2), we predicted the impact of the *SLCO1B1* genotypes on the human pharmacokinetics of OATP1B1 substrates rosuvastatin and repaglinide. In agreement with the expression data, the various genotypes/haplotypes of OATP1B1 had marked effects on the predicted plasma concentration-time profiles of the OATP1B1 substrates rosuvastatin and repaglinide (Fig 6),

translating into reductions in AUC of up to 40% (Fig. 7), when compared with the respective wild types/reference allele.

## DISCUSSION

We quantified the interindividual variability in hepatic expression of OATP1B1, OATP1B3, OATP2B1, and P-gp in human livers (n=64) and cryopreserved hepatocytes (n=12) by LC-MS/MS. OATPs and P-gp expression was measureable in all samples. Consistent with data published by others (Nies et al., 2013), the expression of none of the studied transporters in our liver bank was age (7-70 years) or sex dependent. Since primary human hepatocyte suspension is used to predict the contribution of OATPs to hepatic uptake of drugs in humans, it is interesting to note that the expression of OATPs in human livers was similar to that in cryopreserved hepatocytes (Table 3). However, the expression of MRP2 and BCRP was significantly different ( $1.54 \pm 0.64$  vs  $0.56 \pm 0.21$  and  $0.14 \pm 0.04$  vs  $0.70 \pm 0.22$  fmol/ $\mu$ g membrane protein, respectively ( $P < 0.0001$ ) (Deo et al., 2012; Prasad et al., 2013)). Collectively, our transporter data are comparable or modestly lower than those reported previously in a much smaller sample size (Balogh et al., 2012; Bi et al., 2012; Karlgren et al., 2012; Kimoto et al., 2012; Ohtsuki et al., 2012; Tucker et al., 2012). Based on western blotting, interindividual variability in OATP1B1, OATP1B3, OATP2B1 and P-gp expression have been reported to be 19, 85, 17 (n=117) and 20-fold (n=110), respectively (Meier et al., 2006; Nies et al., 2013). Possible reasons for these differences are differences in tissue procurement method (organ donors vs. resections from diseased livers), subject demographics, membrane fractionation method used, the protein quantification method employed (LC-MS/MS vs immunoblotting), peptides quantified, sample preparation or variation in trypsin digestion efficiencies. Since pure protein standards of the transporters measured are not readily available, the limitation of the peptide-based LC-MS/MS quantification methods of assuming 100% trypsin digestion could be an additional factor. However, since we validated our P-gp expression values using purified human P-gp, these values are not confounded by any methodological issues.

In agreement with clinical data on the effect of genotypes on in vivo activity of OATP1B1 (Mwinyi et al., 2004; Niemi et al., 2004; Voora et al., 2009; Rodrigues et al., 2011; Schipani

et al., 2012; Sortica et al., 2012), we observed that protein expression of OATP1B1 was genotype dependent. SNP c.463C>A is reported to increase lopinavir clearance by 2-fold, possibly due to increased OATP1B1 activity (Hartkoorn et al., 2010). Consistent with this observation, we observed that livers homozygous or heterozygous for this variant contained two-fold higher amounts of OATP1B1 than livers with wild-type alleles. Similarly, SNP c.388A>G increased OATP1B1 expression in human livers compared to the wild type alleles. Clinically, this variant not only increases the clearance of OATP1B1 substrate drugs (e.g. statins) (Mwinyi et al., 2004; Nies et al., 2013), but also increases the efficacy (Rodrigues et al., 2011; Sortica et al., 2012) and reduces the toxicity of statins (Donnelly et al., 2011).

The above analysis did not take into consideration the high degree of linkage disequilibrium between OATP1B1 SNPs. Nies et al. recently reported that *SLCO1B1* haplotype \*14/\*14 is correlated with 28% decrease in atorvastatin AUC relative to the reference allele (\*1a/\*1a). OATP1B1 protein levels were 2-fold higher in individual with the \*14/\*1a haplotype (Nies et al., 2013). Consistent with this report, we observed that OATP1B1 expression in livers with the \*14/\*14 haplotype was 2.2-fold higher than that in livers with the reference haplotype (\*1a/\*1a). Similarly, consistent with previous data (Nies et al., 2013), livers with haplotype \*14/\*1a resulted in 1.9-fold higher protein expression. Importantly, we found that the change in OATP1B1 expression was more pronounced with the 388GG allele vs. the AG allele (Figs. 4 and 5). The presence of c.388GG, but not c.463AA or AC, resulted in a modestly higher OATP1B1 expression than the reference allele (\*1a/\*1a) despite the presence of c.521TC allele which is known to reduce OATP activity and expression (Fig. 5).

OATP1B1 SNP c.521T>C variant is linked to decreased clearance of OATP1B1 substrates (Niemi et al., 2004; Kalliokoski et al., 2008b; Hartkoorn et al., 2010) and is associated with statin-mediated myopathy (Link et al., 2008; Voora et al., 2009). *In vitro* cell surface

biotinylation experiments by Tirona et al. showed that the altered transport activity of the c.521T>C variant, for estrone-3-sulfate or estradiol-17-D-glucuronide, was due to decreased plasma membrane expression of the transporter (Tirona et al., 2001). Although all livers harboring individual SNP c.521TC were not different than wild-type, expression of OATP1B1 was modestly decreased in carriers of SNP c.521TC vs. c.521TT when livers harboring c.388GG were excluded (Fig. 5C)).

Interestingly, we observed that the synonymous *SLCO1B1* SNP c.597C>T was associated with ~1.7 fold increase in hepatic protein expression. However, it is important to note here that SNPs c.388A>G and c.597C>T showed high degree of linkage disequilibrium, thus this may not be an independent effect of the c.597C>T variant. Nevertheless, this observation highlights the fact that quantification of tissue transporter expression can result in the discovery of novel variants resulting in hypothesis-based clinical studies to determine the clinical significance of these variants.

Genetic polymorphism can affect the affinity of a substrate for a transporter ( $K_m$ ) or its maximal transport capacity ( $V_{max}$ ) or both. One factor that can affect  $V_{max}$  is the magnitude of plasma membrane expression of the transporter. Although we measured total membrane expression of OATP1B1, we asked if we could quantitatively predict the *in vivo* consequence of changes in the expression of the transporter using PBPK models. We assumed that OATP1B1 expression data for the various genotypes reflected a proportional change in the plasma membrane expression of the transporter. We predicted up to 40% decrease in the mean AUC of OATP1B1 substrates, rosuvastatin and repaglinide, for the various *SLCO1B1* genotypes (Figs. 6 and 7; Supplemental Table 2). Although the effect of most of these genotypes on the pharmacokinetics of these drugs remains to be tested in the clinic, where available our predictions agreed with the observed data. For example, in agreement with the reported 32% decrease ( $P=0.007$ ) in mean  $AUC_{0-\infty}$  (Kalliokoski et al., 2008a) of repaglinide in carriers of 388GG vs. wild-type, we predicted that AUC changes

from 17.2 (wild-type) to 11.8 ng/ml.h (388GG) (Fig. 7). The latter suggests that despite the fact that we measured the expression of the transporters in total membrane protein (and not specifically in the plasma membrane), we can predict the impact of OATP1B1 genotype on the in vivo disposition of OATP1B1 substrate drugs where the effect appears to occur primarily through change in protein expression. Our predictions also suggest that individuals with \*14/\*14 haplotypes will demonstrate the largest change in the pharmacokinetics of OATP1B1 substrate drugs.

In summary, LC-MS/MS is a sensitive, specific, simple, multiplex approach to simultaneously measure the inter-individual variability in tissue expression of drug transporters. The data presented here as well as those published previously (MRP2 and BCRP) (Deo et al., 2012; Prasad et al., 2013) in the same set of livers indicate that the interindividual variability in the expression of the major drug hepatic transporters is modest (4-8 fold). In addition, expression of these transporters is not associated with age (age 7-70 years) or sex. However, the expression of OATP1B1 was genotype dependent. Our data indicate that measured expression of OATP1B1 in total membranes isolated from liver tissue (vs. plasma membrane) was predictive of the in vivo consequences of OATP1B1 genotype on drug pharmacokinetics. Collectively, the data presented here will potentially allow us to quantitatively predict transporter-based drug disposition and DDI through population PBPK modeling.

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## **Authorship contributions**

Participated in research design: Prasad, Evers, Gupta, Salphati, Hop and Unadkat

Conducted experiments: Prasad, Shukla

Performed data analysis: Prasad and Unadkat

Wrote or contributed to the writing of the manuscript: Prasad, Evers, Gupta, Salphati, Hop,  
Shukla, Ambudkar and Unadkat

Contributed new reagents: Evers, Gupta, Salphati, Hop, Shukla and Ambudkar

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## Footnotes

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## Figure legends

**Fig. 1.** Interindividual variability and mean  $\pm$  SD (last bar) in hepatic protein expression of OATP1B1 (A), OATP1B3 (B), OATP2B1 (C) or P-gp (D) in liver samples.

**Fig. 2.** Hepatic protein expression of OATP1B1 (A), OATP1B3 (B), OATP2B1 (C) or P-gp (D) does not correlate with age ( $p < 0.05$ ).

**Fig. 3.** Hepatic protein expression of OATP1B1 (A), OATP1B3 (B), OATP2B1 (C) or P-gp (D) is not dependent on sex.

**Fig. 4.** OATP1B1 protein expression based on individual alleles (% frequencies in parenthesis). Horizontal line: median; +: mean value; boxes: 25th-75th percentiles; whiskers: non-outlier range. Same letters indicate significant difference;  $P < 0.0001$  (a, b),  $P < 0.005$  (c).

**Fig. 5.** (A) *SLCO1B1* haplotype classification based on the presence of the three previously described key variants; (B) Hepatic OATP1B1 protein expression is haplotype dependent. *SLCO1B1* haplotype (% frequencies in parenthesis) are arranged by median OATP1B1 protein expression; (C) When carriers of c.388GG were excluded, OATP1B1 protein expression in livers harboring c.521TC allele was moderately but significantly lower than those harboring the TT allele. OATP1B1 protein expression in livers harboring c.388GG (but not SNP c.463CA or AA) was moderately but significantly higher than those harboring the AA allele. Horizontal line: median; +: mean; boxes: 25th-75th percentiles; whiskers: non-outlier range. Same letters indicate significant difference;  $P < 0.0001$  (a, b, c, d, e, f) and  $P < 0.05$  (g)

**Fig. 6.** Mean rosuvastatin (A) and repaglinide (B) plasma concentration-time profiles (po) in populations harboring OATP1B1 wild-type (\*1a/\*1a, black lines) or \*14/\*14 (gray lines) haplotypes. The 90% confidence intervals around the mean are represented by the dashed and dotted lines respectively.

**Fig. 7.** Simcyp predicted mean area under the curve (AUC) of rosuvastatin (A) or repaglinide (B) in virtual populations harboring different *SLCO1B1* genotypes or haplotypes. Error bars show 90% confidence intervals around the mean.

**Tables 1:** MRM parameters of peptides selected for targeted analysis of OATP1B1, OATP1B3, and P-gp. Labeled amino acid residues are shown in bold and italic.

Transporter	Peptide	Residues	Parent ion (z=2)	Product ions			(z=1)	
				1	2	3	Frag (V)	CE (eV)
OATP1B1	NVTGFFQSFK	321-330	587.9	961.4	860.5	803.2	125	13
(Peptide 1)	NVTGFFQSFK		591.9	969.5	868.5	811.2	125	14
OATP1B1	YVEQQYGQPSSK	362-373	707.5	766.5	418.3	1152.5	155	22
(Peptide 2)	YVEQQYGQPSSK		711.5	774.5	426.3	1160.5	155	22
OATP1B3	NVTGFFQSLK	321-330	570.8	927.5	826.3	622.3	130	14
(Peptide 1)	NVTGFFQSLK		574.8	935.6	834.3	630.3	130	14
OATP1B3	IYNSVFFGR	615-623	551.8	826.5	712.5	526.6	135	13
(Peptide 2)	IYNSVFFGR		556.8	836.6	635.2	536.6	135	13
OATP2B1	VLAVTDSPAR	314-323	514.8	816.4	846.4	745.3	130	13
(Peptide 1)	VLAVTDSPAR		519.9	826.4	856.4	755.3	125	13
OATP2B1	SSPAVEQQLLVSGPGK	687-702	799.0	712.3	1026.3	1155.3	180	20
(Peptide 2)	SSPAVEQQLLVSGPGK		803.0	1163.3	1034.3	715.9	180	20
P-gp	NTTGALTTR	809-817	467.8	618.3	719.4	820.5	125	12
(Peptide 1)	NTTGALTTR		472.8	628.3	729.5	830.5	125	12
P-gp	IATEAIENFR	896-905	582.3	749.5	678.5	979.5	130	16
(Peptide 2)	IATEAIENFR		587.3	759.5	688.5	989.5	130	16

Frag= fragmentor and CE= collision energy

**Table 2:** Characteristics of cryopreserved hepatocytes purchased from commercial sources

Lot number	Source	Sex	Age (years)	Race	Number of subjects
AZL	Celsis, In vitro Technologies	NA	33-88	18 C, 1 B and 1 H	20
PQP	Celsis, In vitro Technologies	10 M and 10 F	17-75	16 C, 2 B and 2 H	20
OFY	Celsis, In vitro Technologies		17-65	8 C, 1 B and 1 H	10
IRK	Celsis, In vitro Technologies	5 M and 5 F	19-80	9 C and 1 B	10
UMJ	Celsis, In vitro Technologies	5 M and 5 F	2-76	9 C and 1 H	10
Hu4193	Invitrogen	M	42	C	1
4524339002	BD Gentest	F	64	C	1
305	BD Gentest	M	67	C	1
4535888003	BD Gentest	M	14	C	1
4534583002	BD Gentest	M	11 (months)	H	1
512	Xenotech	F	62	C	1
706	Xenotech	F	53	C	1

NA= not available; M=male; F=female; C=Caucasian; B=Black; H=Hispanic

**Table 3:** Mean  $\pm$  SD (range, i.e, maximum/minimum, in parentheses) protein expression in human livers and human hepatocytes observed in this study (bolded) or reported by others

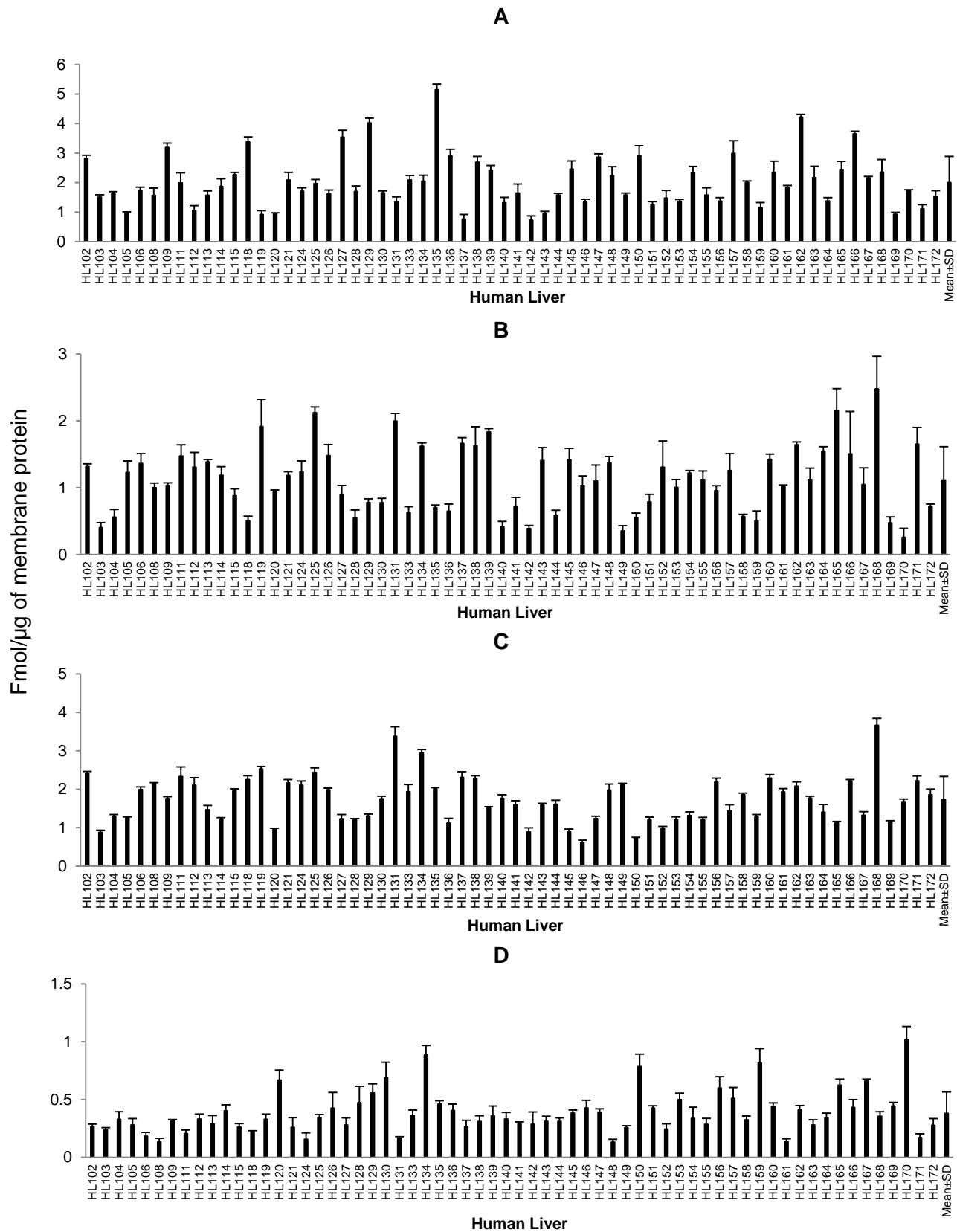
Number of samples	OATP1B1	OATP1B3	OATP2B1	P-gp	Reference
<b>Liver tissue (fmol/<math>\mu</math>g membrane protein)</b>					
<b>n=64 (Peptide 1)</b>	<b>2.0 <math>\pm</math> 0.9 (7)</b>	<b>1.1 <math>\pm</math> 0.5 (8)</b>	<b>1.7 <math>\pm</math> 0.6 (5)</b>	<b>0.4 <math>\pm</math> 0.2 (8)</b>	<b>This study</b>
<b>n=64 (Peptide 2)</b>	NQ	<b>0.9 <math>\pm</math> 0.4 (8)</b>	<b>1.5 <math>\pm</math> 0.6 (6)</b>	<b>0.3 <math>\pm</math> 0.2 (7)</b>	<b>This study</b>
n=17	2.7 $\pm$ 3.7*	1.7 $\pm$ 0.5	0.5 $\pm$ 0.9	1.5 $\pm$ 0.4	(Ohtsuki et al., 2012)
n=9	9.7 $\pm$ 4.3	6.3 $\pm$ 2.8	3.7 $\pm$ 1.4	NQ	(Kimoto et al., 2012)
n=13	NQ	NQ	NQ	0.7 $\pm$ 0.3	(Tucker et al., 2012)
n=12	7.2 $\pm$ 0.3	6.3 $\pm$ 0.4	4.0 $\pm$ 0.4	NQ	(Karlgrén et al., 2012)
n=4	10.6 $\pm$ 4.6	5.9 $\pm$ 3.8	2.9 $\pm$ 1.3	NQ	(Balogh et al., 2012)
<b>Cryopreserved suspended hepatocytes</b>					
<b>n=12<sup>s</sup></b>	<b>2.4 <math>\pm</math> 0.5 (2)</b>	<b>0.9 <math>\pm</math> 0.5 (5)</b>	<b>1.7 <math>\pm</math> 0.5 (3)</b>	<b>0.5 <math>\pm</math> 0.1 (4)</b>	<b>This study</b>
<b>n=12</b>	<b>NQ</b>	<b>0.7 <math>\pm</math> 0.3 (4)</b>	<b>1.3 <math>\pm</math> 0.5 (3)</b>	<b>0.4 <math>\pm</math> 0.1 (4)</b>	<b>This study</b>
n=14	4.9 $\pm$ 1.9	1.7 $\pm$ 0.6	1.6 $\pm$ 0.5	NQ	(Kimoto et al., 2012)
n=1	5.8	5.8	4.1	NQ	(Ji et al., 2012)
n=1	3.4	1.5	1.8	NQ	(Bi et al., 2012)
<b>Sandwich-cultured hepatocytes</b>					
n=3	5.4 $\pm$ 1.0	4.3 $\pm$ 0.2	2.9 $\pm$ 2.5	13.9 $\pm$ 11.8	(Schaefer et al., 2012)
n=5	5.8 $\pm$ 3.3	0.8 $\pm$ 0.3	1.0 $\pm$ 0.2	NQ	(Kimoto et al., 2012)
n=1	5.3	0.9	1.2	NQ	(Bi et al., 2012)

NQ – not quantified; \* OATP1B1 was detected in only 8 liver tissue samples. <sup>s</sup>7 batches of individual and 5 batches of pooled hepatocytes (See Table 2)

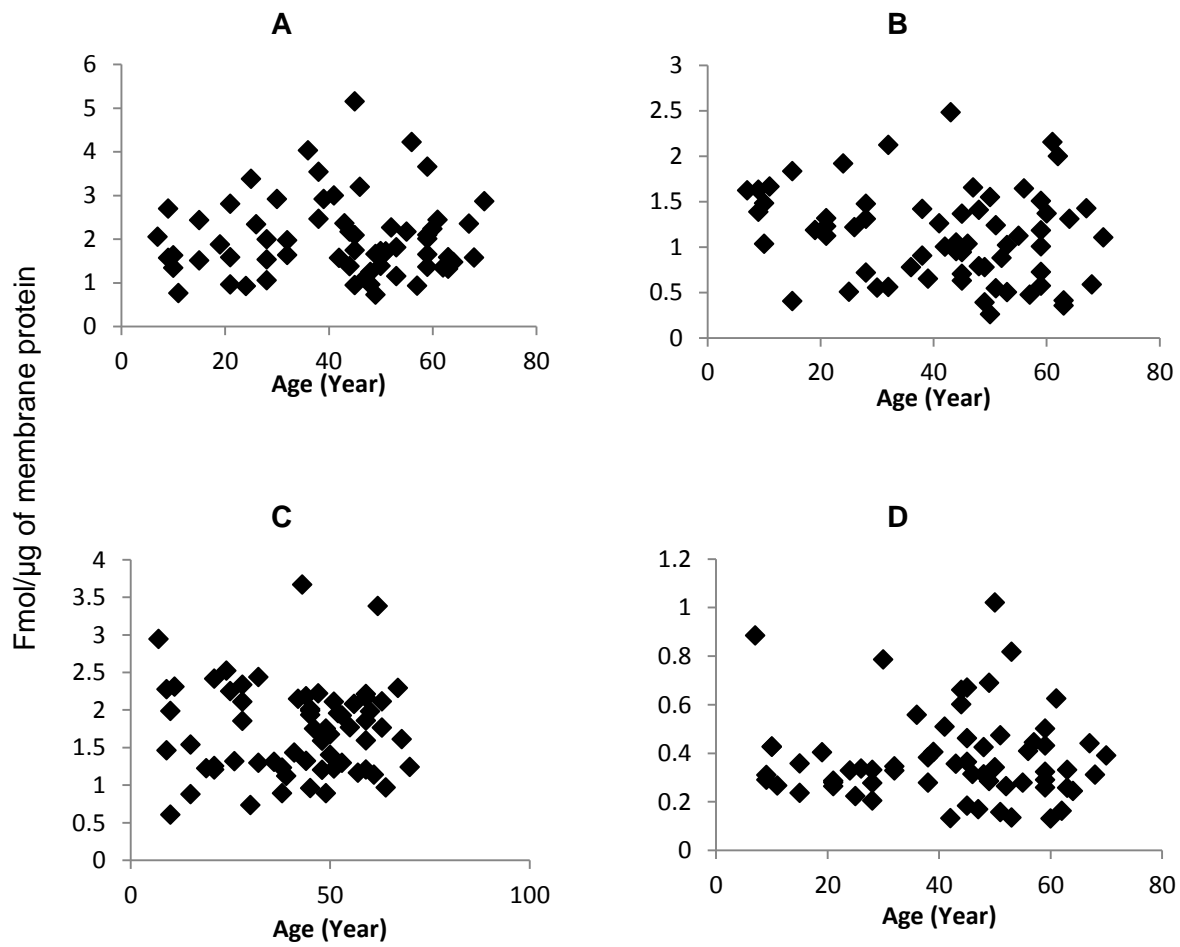
**Table 4:** Frequency of OATP1B1, OATP1B3 and P-gp SNPs detected in the UW liver bank

Marker ID	Variant	Change for variant	Frequency in UW liver bank		
			Homozygous variant	Heterozygous variant	Wild-type
OATP1B1					
rs4149015	-11187G>A	Promoter	0	9	53
rs2306283	388A>G	N130D	10	30	22
rs11045819	463C>A	P155T	4	9	49
rs4149056	521T>C	V174A	1	21	40
rs4149057	571T>C	L191L	16	34	12
rs2291075	597C>T	F199F	10	33	19
OATP1B3					
rs4149117	334G>T	A112S	0	45	17
rs7311358	699A>G	I233M	0	45	17
rs2053098	1557G>A	A519A	0	45	17
rs3764006	1833A>G	G611G	0	42	20
P-gp					
rs2214102	-1G>A	5'UTR	0	6	56
rs2235015	287-25G>T	Intron	0	19	43
rs10276036	IVS9-44A>G	Intron	19	31	12
rs1128503	1236C>T	G412G	18	33	11
rs2032588	1350+44C>T	Intron	0	7	55
rs2235033	1554+24C>T	Intron	22	32	8
rs2235013	1725+38A>G	Intron	21	33	8
rs9282564	61A>G	N21D	7	11	45
rs2235040	2481+24G>A	Intron	0	12	50
rs2032582	2677G>T, 2677G>A	A893S, A893T	17	32	13
rs3213619	-129T>C	5'UTR	0	4	58
rs1045642	3435C>T	I1145I	22	30	10
rs17064	*89A>T	3'UTR	0	7	55
rs3842	*193A>G	3'UTR	0	18	44

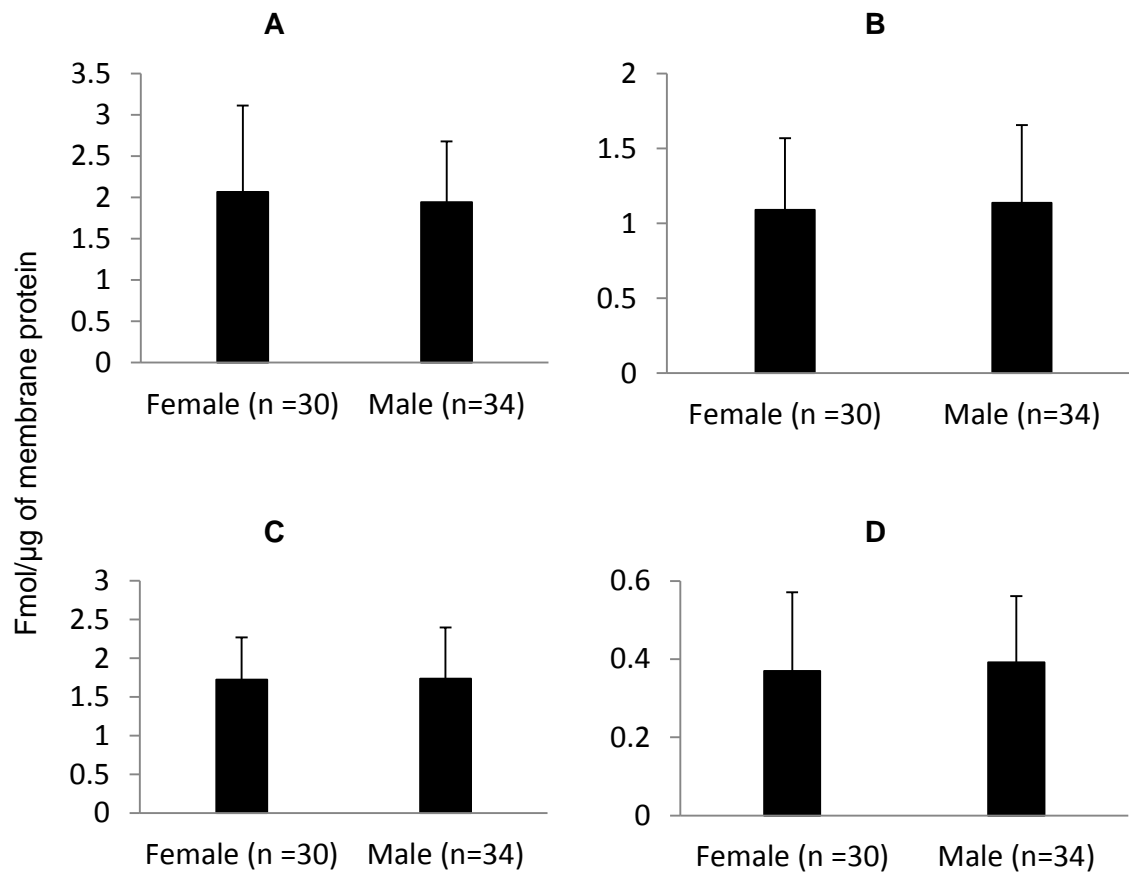
OATP2B1 SNPs, rs59305495, rs72559740, rs1109407, rs1621378 and rs2306167 were not found in the UW liver bank. All other SNPs available in the Affymetrix DMET Plus array for OATP1B1, OATP1B3 and P-gp were not observed.



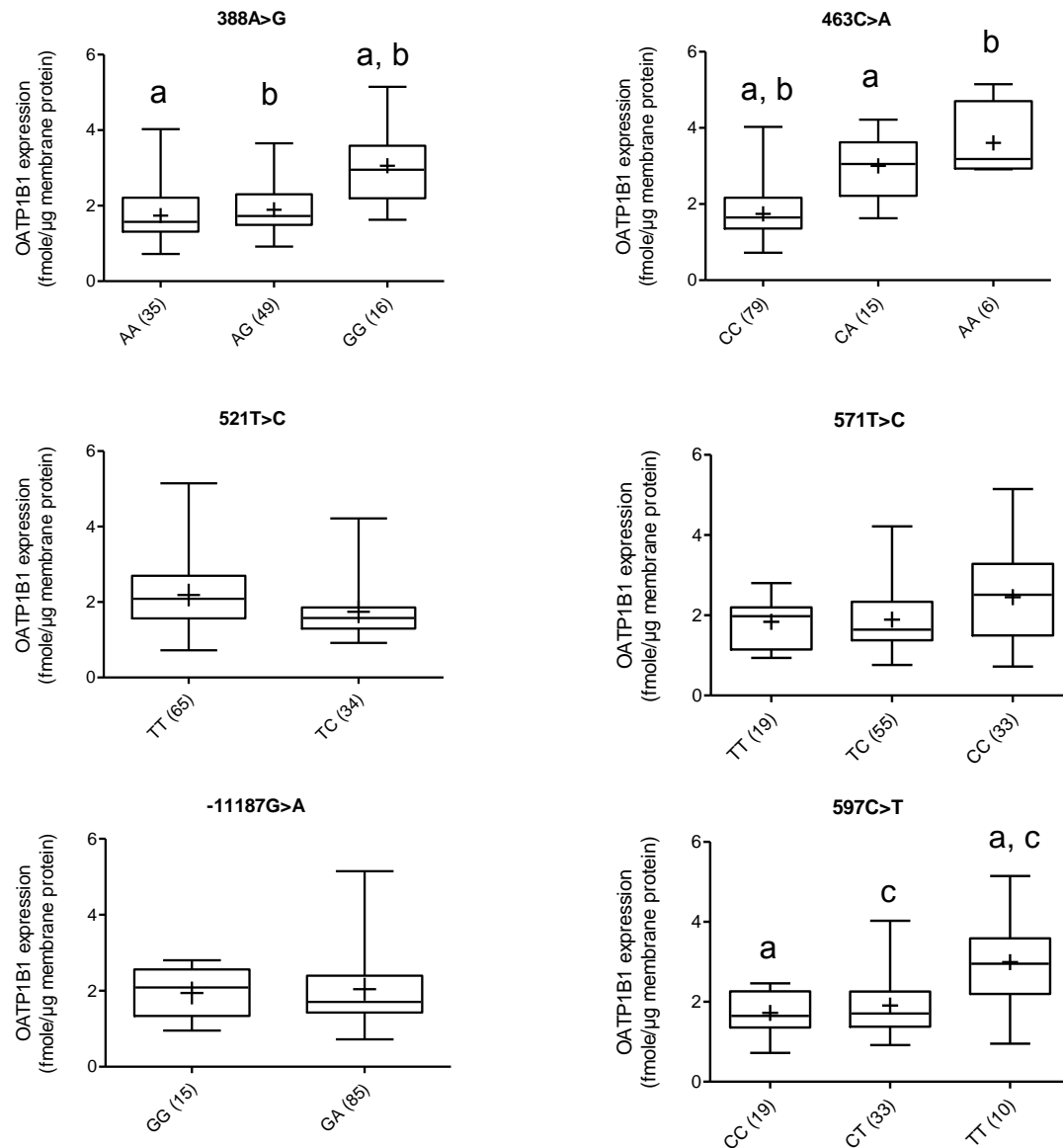
**Fig. 1**



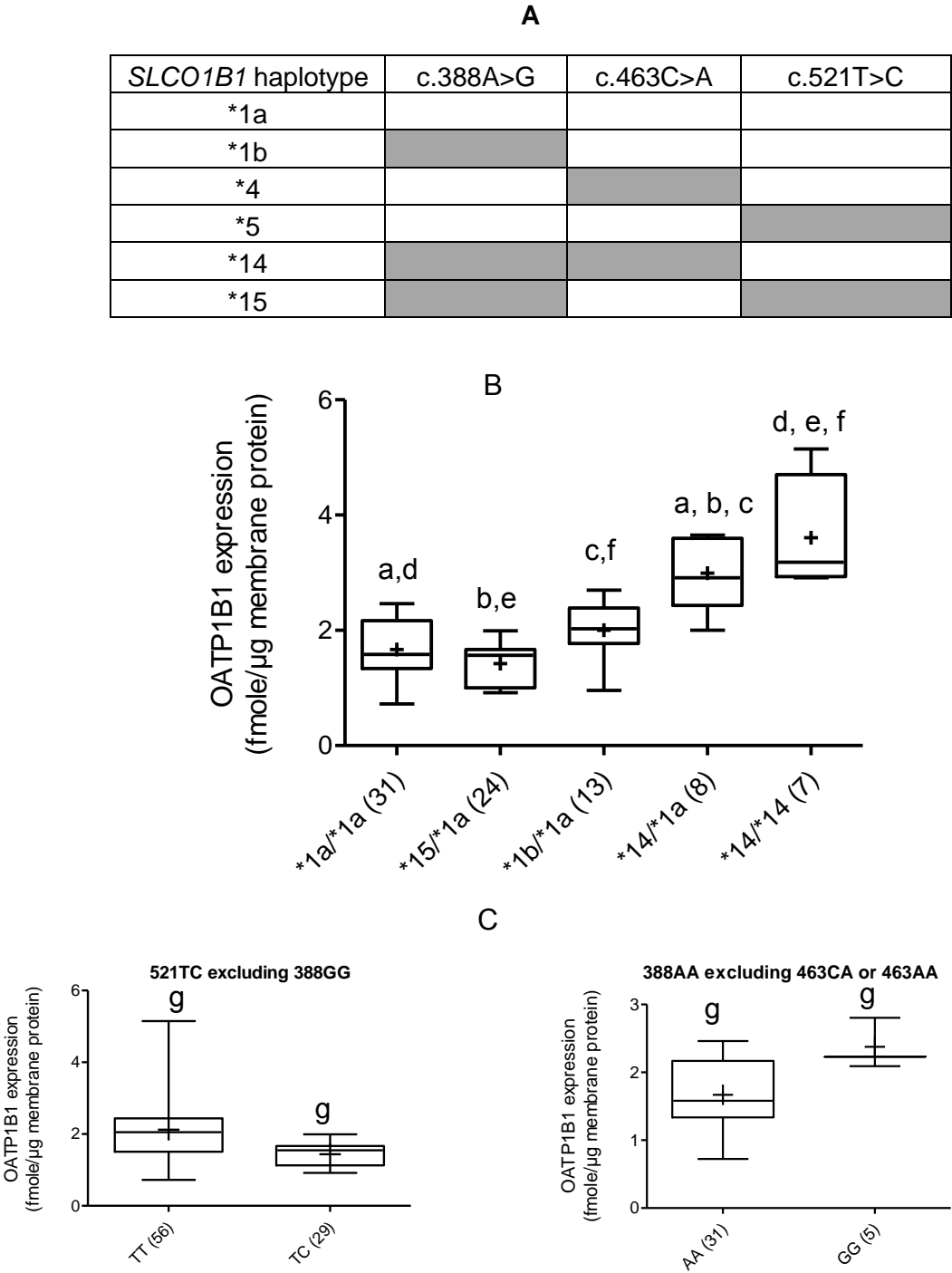
**Fig. 2.**



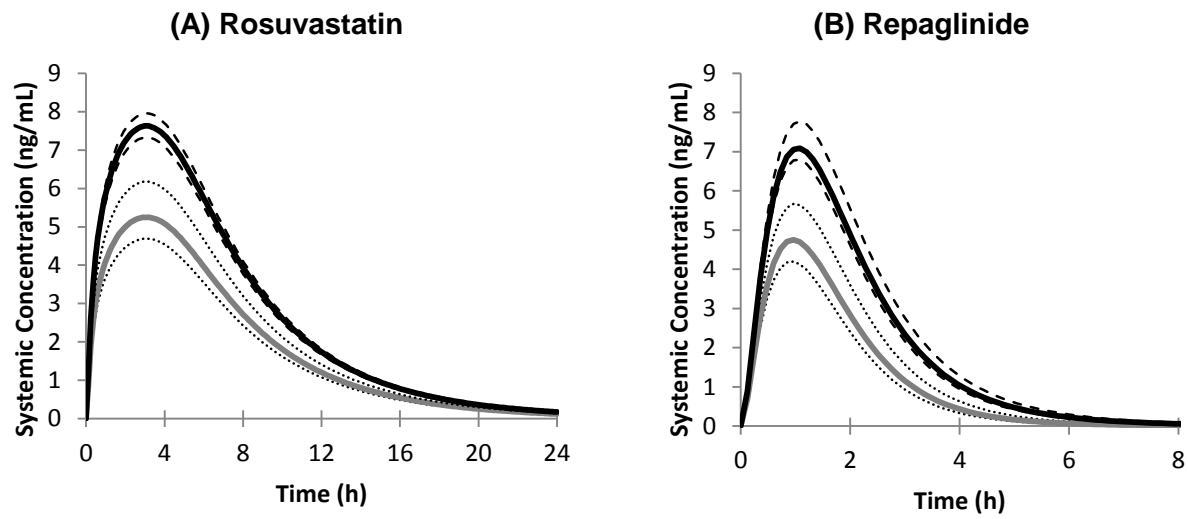
**Fig. 3.**



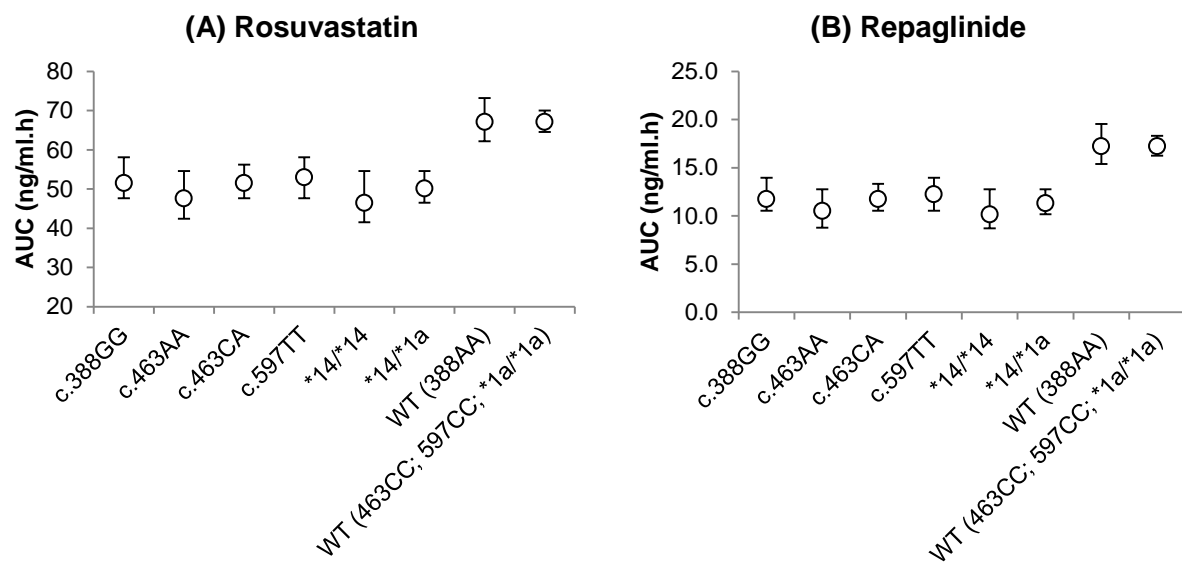
**Fig. 4**



**Fig. 5.**



**Fig. 6**



**Fig. 7**