Proteomics of human liver membrane transporters: a focus on fetuses and newborn infants

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ABSTRACT

Background: Hepatic membrane transporters are involved in the transport of many endogenous and exogenous compounds, including drugs. We aimed to study the relation of age with absolute transporter protein expression in a cohort of 62 mainly fetus and newborn samples.

Methods: Protein expressions of BCRP, BSEP, GLUT1, MCT1, MDR1, MRP1, MRP2, MRP3, NTCP, OCT1, OATP1B1, OATP1B3, OATP2B1 and ATP1A1 were quantified with LC-MS/MS in isolated crude membrane fractions of snap-frozen post mortem fetal and pediatric, and surgical adult liver samples. mRNA expression was quantified using RNA sequencing, and genetic variants with TaqMan assays. We explored relationships between protein expression and age (gestational age [GA], postnatal age [PNA], and postmenstrual age); between protein and mRNA expression; and between protein expression and genotype.

Results: We analyzed 36 fetal (median GA 23.4 weeks [range 15.3-41.3]), 12 premature newborn (GA 30.2 weeks [24.9-36.7], PNA 1.0 weeks [0.14-11.4]), 10 term newborn (GA 40.0 weeks [39.7-41.3], PNA 3.9 weeks [0.3-18.1]), 4 pediatric (PNA 4.1 years [1.1-7.4]) and 8 adult liver samples. A relationship with age was found for BSEP, BCRP, GLUT1, MDR1, MRP1, MRP2, MRP3, NTCP and OATP1B1, with the strongest relationship for postmenstrual age. For most transporters mRNA and protein expression were not correlated. No genotype-protein expression relationship was detected.

Discussion and conclusion: Various developmental patterns of protein expression of 13 hepatic transporters emerged in fetuses and newborns up to four months of age. Postmenstrual age was the most robust factor predicting transporter expression in this cohort. Our data fill an important gap in current pediatric transporter ontogeny knowledge.

INTRODUCTION

Membrane-embedded transporter proteins are crucial in handling endogenous and exogenous compounds. More specifically, hepatic transporters are critical determinants in drug distribution, metabolism and biliary secretion, as they facilitate influx and efflux of substrates from hepatocytes, where metabolism takes place.[1]

Children admitted to a neonatal or pediatric intensive care unit may receive many drugs. Earlier it was shown that infants with normal weight received on average four different drugs, and infants with an extreme low birth weight, often prematurely born, up to 17 drugs.[2] Many of these drugs are substrates for transporters[3], and the expression and activity of certain transporters are known to be subject to age-related changes.[1] An example of a transporter substrate is morphine, which is widely used in newborns and children. Morphine is taken up into the hepatocyte by the transporter OCT1, where it is glucuronidated mainly by UGT2B7.[4] Data suggest lower protein expression of hepatic OCT1 in younger age groups[5,6], leading to elevated plasma levels and therefore posing a higher risk of adverse events like respiratory depression. However, the exact developmental pattern of OCT1 in fetuses and premature newborns is not known, while data for other transporters are also scarce or even lacking.[1,3]

In neonates and young infants, age can be defined in various ways: gestational age (GA) - reflecting duration of pregnancy at birth; postnatal age (PNA) – the age after birth; and postmenstrual age (PMA) – the combination of gestational age and postnatal age. Both GA and birth are important determinants of postnatal gene expression of drug metabolizing enzymes.[7] We hypothesize that this also accounts for drug transporters. More insight in the relative importance of these determinants could help personalize drug dosing in this young vulnerable population.

Previously, we explored the hepatic protein expressions of 10 clinically relevant transporters in 25 liver samples from fetuses, neonates and young infants.[8] Protein expression of a number of these transporters was related to age, and important transporter-specific differences were found. While this exploratory study was clearly informative, the sample size was too small to define transporter-specific maturational patterns. A recent publication from Prasad et al. describes the postnatal ontogeny of hepatic drug transporters in a wider cohort, but the younger ages (<four months) were not well represented.[6] Data on gene expression of transporters in the younger ages are richer[1,8], but lack of correlation between gene and protein expression restricts us from extrapolating these findings. Thus, knowledge of transporter protein expression is lacking for fetuses and ages up to 18 weeks PNA.

Besides ontogeny, drug transporter expression and activity can be influenced by genetic variants, as described in adults.[9] For *SLC22A1*/OCT1 a relationship with genotype was suggested by variation in the pharmacokinetics of tramadol, an OCT1 substrate, in preterm infants, even when *in vitro* data suggested developmentally low expression.[10] This is interesting as for some drug metabolizing enzymes the interplay between development and genetics obscures an effect of genetic variation. But pediatric clinical data for transporters substrates are scarce.

In the current study we aimed to elucidate the developmental expression patterns of various hepatic drug transporters in an expanded cohort of mainly fetal and newborn samples up to 18 weeks PNA, also including the samples from our previous pilot study. The large variation in GA and PNA in this cohort enabled us to analyze whether PNA or PMA correlates strongest with transporter expression. We also investigated correlation between protein expression and mRNA expression in a subset of this cohort, and analyzed whether genotype, in addition to age, explains the variability in expression of hepatic drug transporters. Expression patterns were compared to hepatic transporter proteins in stably transfected cell lines (HEK293 cells expressing OATP1B1, OATP1B3, or OCT1 and MDCKII cells expressing MDR1, MRP2 or BCRP) in order to be used for future PBPK modeling.

MATERIAL AND METHODS

Tissue samples

Post-mortem liver tissue samples from autopsy of fetuses (from therapeutic abortions or stillbirths) and infants were provided by the Erasmus MC Tissue Bank. Tissue was procured at the time of autopsy within 24 hours after death and snap-frozen at -80^{0}C for later research use. The Erasmus MC Research Ethics Board waived the need for formal ethics approval according to the Dutch Law on Medical Research in Humans. Tissue was collected when parental written informed consent for both autopsy and the explicit use of the tissue for research was present. The samples were selected when the clinical diagnosis of the patient was not related to hepatic problems and the tissue was histologically normal (Supplemental Table 1).

Human adult liver tissue samples were a gift from Prof. G.M.M. Groothuis (University of Groningen, Groningen, the Netherlands) (n=3) and Prof. P. Artursson (Uppsala University, Uppsala, Sweden) (n=5). These had been collected anonymously as surgical waste material after partial hepatectomy because of liver metastasis. For these samples, a no-

objection clause permitted use for research purposes in line with the Dutch guidelines on secondary use of human tissue.

Selection of hepatic transporters

Thirteen clinically relevant hepatic transporters were selected (*gene name*/protein name): breast cancer resistance protein (*ABCG2*/BCRP), bile salt export pump (*ABCB11*/BSEP), glucose transporter 1 (*SLC2A1*/GLUT1), monocarboxylate transporter 1 (*SLC16A1*/MCT1), multidrug resistance protein 1 (*ABCB1*/MDR1), multidrug resistance associated protein (*ABCC*/MRP) 1, 2 and 3, sodium-taurocholate cotransporting polypeptide (*SLC10A1*/NTCP), organic anion-transporting polypeptide (*SLCO*/OATP) 1B1, 1B3 and 2B1, and organic cation transporter 1 (*SLC22A1*/OCT1). Analysis on the transporters MRP1, NTCP, OATP1B3 and OCT1 was lacking in our pilot study[8], but was added in this expanded study because of their clinical relevance. We also selected ATP1A1, which is often used as a housekeeping protein.[6]

Protein expression

Absolute transporter protein expression of the selected hepatic drug transporters was quantified in crude membrane fractions in all samples, including the samples from our pilot-study, using LC-MS/MS as previously described[11], with some minor modifications regarding isolation of the membrane fractions (see below). Crude membrane fractions include nuclei, mitochondria as well as the microsomal and plasma membranes. Absolute transporter expression was also determined in cell pellets of HEK-OATP1B1, -OATP1B3, -OCT1, MDCKII-MDR1, -MRP2, and –BCRP cells.

Isolation of crude membrane fractions from tissue samples was conducted as follows. Approximately 10 mg liver tissue or approximately 20 x 10^6 cells was homogenized in a hypotonic buffer (0.5 mM sodium phosphate, 0.1 mM EDTA, and a cocktail of protease inhibitors containing 2 mM phenylmethylsulfonylfluoride, aprotinin, leupeptin, and pepstatin) using a Potter-Elvehjem homogenizer. The homogenate was centrifuged at 100,000 g for 30 min at 4°C using a LE-80k Centrifuge with an SW28 rotor (Beckman Coulter, Fullerton, CA, USA). This step was repeated, and the remaining pellet containing the crude membrane fraction was resuspended in 200 µL of isotonic buffer (10 mM Tris-HEPES and 250 mM sucrose (pH 7.4)). A maximum of 100 µg of crude membrane protein was used for tryptic digestion. Samples were diluted with 2 volumes of 90% methanol. The proteins were subsequently reduced with 0.01 M dithiothreitol at 37°C for 60 minutes and alkylated with 0.04 M iodoacetamide for 20 minutes at room temperature in the dark. Digestion was performed after addition of $CaCl_2$ (final concentration 1 nM) and 0.5 mg trypsin in 17% methanol by diluting the solution with 50 mM NH_4HCO_3. After overnight incubation the samples were incubated for another 2 hours with 0.5

mg trypsin to ensure complete digestion of the protein sample. The efficiency of the tryptic digestion using this protocol was previously checked using SDS-PAGE followed by silver stain, confirming complete digestion.[11] Finally the protein digests were evaporated by vacuum centrifugation (Scanvac, Ballerup, DK) and dissolved in 100 ml 15% acetonitrile containing 0.1% formic acid and 5 ng ml^{-1} internal standard (AQUA peptide mix, Supplemental Table 2). Samples were analyzed using an ultraperformance liquid chromatography coupled to a 6500 QTrap mass spectrometer (AB Sciex, Nieuwerkerk aan den IJssel, the Netherlands). Multiple reaction monitoring transitions were determined from tandem mass spectra, obtained by direct infusion of 0.5 mg mL^{-1}. Per peptide, three transitions were chosen (Q3-1, Q3-2, and Q3-3) for quantification and confirmation. A peptide labeled with ^{15}N and ^{13}C (AQUA peptide) was synthesized (Sigma-Aldrich, Steinheim, DE) and used as an internal standard for quantification (Supplemental Table 2). Peak identification and quantification were performed using Analyst software version 1.6.

mRNA expression

mRNA expression of the selected drug transporters was determined in a subset of 31 samples using RNA-Sequencing (RNA-Seq). RNA was isolated from hepatic tissue using QiaSchredder column and RNeasy Mini kit (both Qiagen, Valencia, CA) as described by Mooij et al [12]. Samples with an RNA integrity number of <5 were excluded. The RNA-Seq experiments were performed according to the Illumina RNA-Seq protocol (San Diego, CA). In brief, a population of poly(A)$^+$ mRNA was selected and converted to a library of cDNA fragments (220–450 bp) with adaptors attached to both ends, using an Illumina mRNA-Seq sample preparation kit. The quality of the library preparation was confirmed by analysis on a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). The cDNA fragments were then sequenced on an Illumina HiSeq 2000 to obtain 100-bp sequences from both ends (paired end). The resulting reads were mapped by Bowtie 2[13] to the transcriptome constructed through annotated genes/transcripts according to the reference human genome GRCh37.61/hg19. The mapped reads were then assigned to transcripts from which the expression of each transcript is estimated by RSEM.[14] The counts of RNA-Seq fragments were used to indicate the amount of identified mRNA transcripts, presented in transcripts per million (TPM).[14]

For each transporter we calculated the total TPM values of all mRNA transcripts, and the TPM values of only the mRNA transcripts coding for a full functioning protein (Ensembl genome database). Correlation with protein levels as determined in the same sample was tested with Spearman's rank correlation coefficient.

Genetics

Single nucleotide polymorphisms (SNPs) were only selected when mRNA and/or protein expression of our selection of transporters was expected to be influenced, based on information in the PharmGKB database.[15] Liver samples of children were genotyped for these SNPs (Supplemental Table 3). Next, within a particular genotype the effect of age was studied. Adult samples were not genotyped for logistic reasons. Because previously the influence of diplotypes of *SLCO1B1* on protein expression was shown[9], we studied relationships between *SLCO1B1* *1A, *1B, *4, *5, *14 and *15 and protein expression.

DNA was isolated from liver tissue according to protocol using the DNeasy® Blood and Tissue Kit (Qiagen, Valencia, CA). DNA concentrations were measured on the Nanodrop® 1000 Spectrophotometer (Thermo Fisher Scientific®). The DNA isolates were diluted in 1X TE Buffer to a 10 ng μL^{-1} solution for SNP analysis. The SNPs were genotyped according to the TaqMan® allelic discrimination assays. The PCR program consisted of an initial denaturation and DNA polymerase activation step at 92°C for 20 s, followed by 40 cycles at 95°C for 3 s and 60°C for 30 s. All PCR reactions and post-PCR detection were performed on a 7500 Fast Real-Time PCR System (software version 2.3; Applied Biosystems).

Cell lines

HEK293 cells overexpressing *SLCO1B1* *1A (NM_006446.4 referring to wild-type; hereafter named *SLCO1B1*) or *SLCO1B3* (NM_019844.3) were generated as previously described by our group[11,16]. HEK293 cells, stably overexpressing *SLC22A1* (NM_003057.2), were generated in a similar way, by transfection with pIRES puro-OCT1 (internally designed, produced by Baseclear, Leiden, NL), applying puromycin selection pressure and selecting colonies for further analysis. MDCKII cells stably overexpressing MDR1, MRP2 or BCRP were licensed from The Netherlands Cancer Institute (NKI, Amsterdam).[17-19]

Data and statistical analysis

Data are expressed as median (range), unless otherwise stated. The relationship of age with protein expression levels was studied as follows: first, differences in expression between age groups were explored. We distinguished five age groups: fetal, premature newborn (GA <37 weeks; PNA 0 – 18 weeks), term newborn (GA >37 weeks, PNA 0 – 18 weeks), pediatric (1.5 – 18 year) and adult liver samples. Next, in the combined first three age groups (further referred to as fetal/newborn cohort) the correlation between age on a continuous scale (GA, PNA and PMA) and protein levels was assessed. Within a particular genotype the effect of age on transporter protein expression was studied as above. Relationship between mRNA expression and protein expression were studied with correlation.

Kruskal-Wallis tests with Dunn's post-hoc test were used for multiple comparisons between age groups, and Spearman's rank correlation coefficient was used for testing correlations. Influence of gender on transporter protein expression was tested with a Mann-Whitney U test. A two-sided significance level of $p<0.05$ is used throughout the paper. For Dunn's post-hoc test for multiple comparisons the adjusted p-values are reported, in which a correction for multiple testing for age groups is applied. Statistical analyses were performed using IBM SPSS Statistics software (SPSS Statistics for Windows, version 21.0; IBM, Armonk, NY).

RESULTS

Descriptive results

In total 71 hepatic tissue samples were available for the study, including 25 samples of our pilot study.[8] One sample was detected as an outlier due to inexplicably high transporter expression and was excluded. See Table 1 for the age distribution. Gender was known for the pediatric samples only: 35 male and 27 female. The Tissue Bank provided only the following clinical data: GA, PNA, gender, and main clinical diagnosis. The adult tissue was histologically normal tissue and no additional clinical data were available, due to the anonymous sample collection.

Table 1 Age distribution of study samples in each age group.

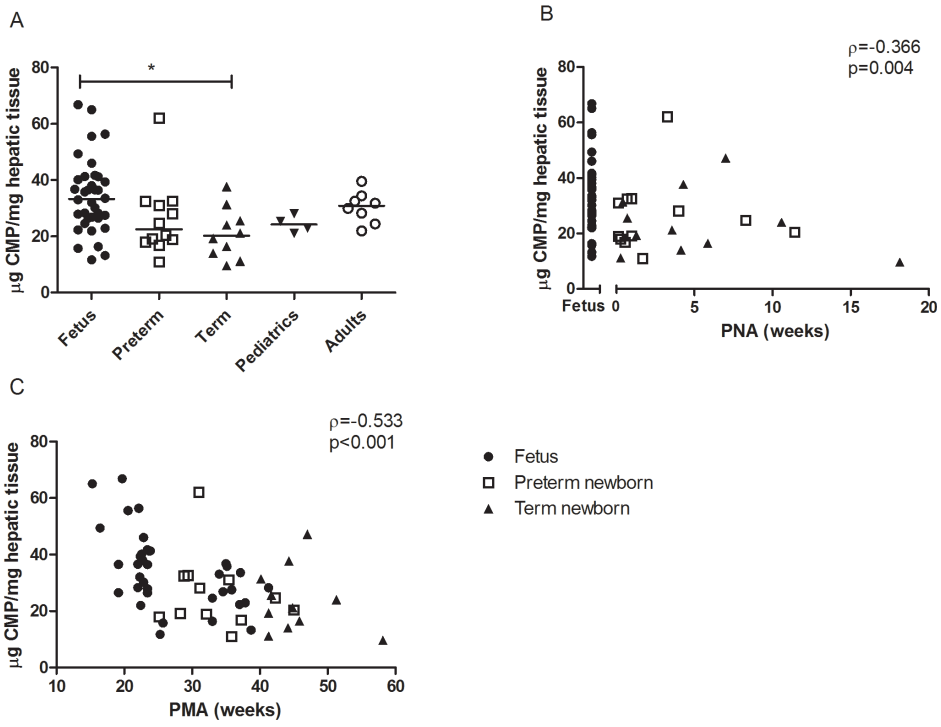
		All	Fetuses	Preterm newborns	Term newborns	Pediatrics	Adults
Age distribution	GA	NA	23.4 (15.3-41.3) weeks	30.2 (24.9-36.7) weeks	40.0 (39.7-41.3) weeks	NA	NA
	PNA	NA	NA	1.0 (0.14-11.4) weeks	3.86 (0.29-18.1) weeks	4.13 (1.08-7.44) years	NA

GA=gestational age, PNA=postnatal age, NA=not available.

Protein expression

The selected hepatic transporter proteins were detected in nearly all samples; in two samples MRP2 could not be detected. There was high variability in expression between transporters and between individual samples (Supplemental Table 4). Protein expression in males and females was similar (Supplemental Table 5). Crude membrane protein yield per mg tissue was higher in fetuses than in term newborns (Figure 1A). Moreover, it was negatively correlated with PNA and PMA in the fetal/newborn cohort (Figure 1B and 1C, respectively).

Figure 1 Crude membrane protein (CMP) yield per amount of hepatic tissue, presented for various age groups (A), and, for the fetal/newborn cohort, for postnatal age (PNA) (B) and postmenstrual age (PMA) (C). ρ=Spearmans rho.



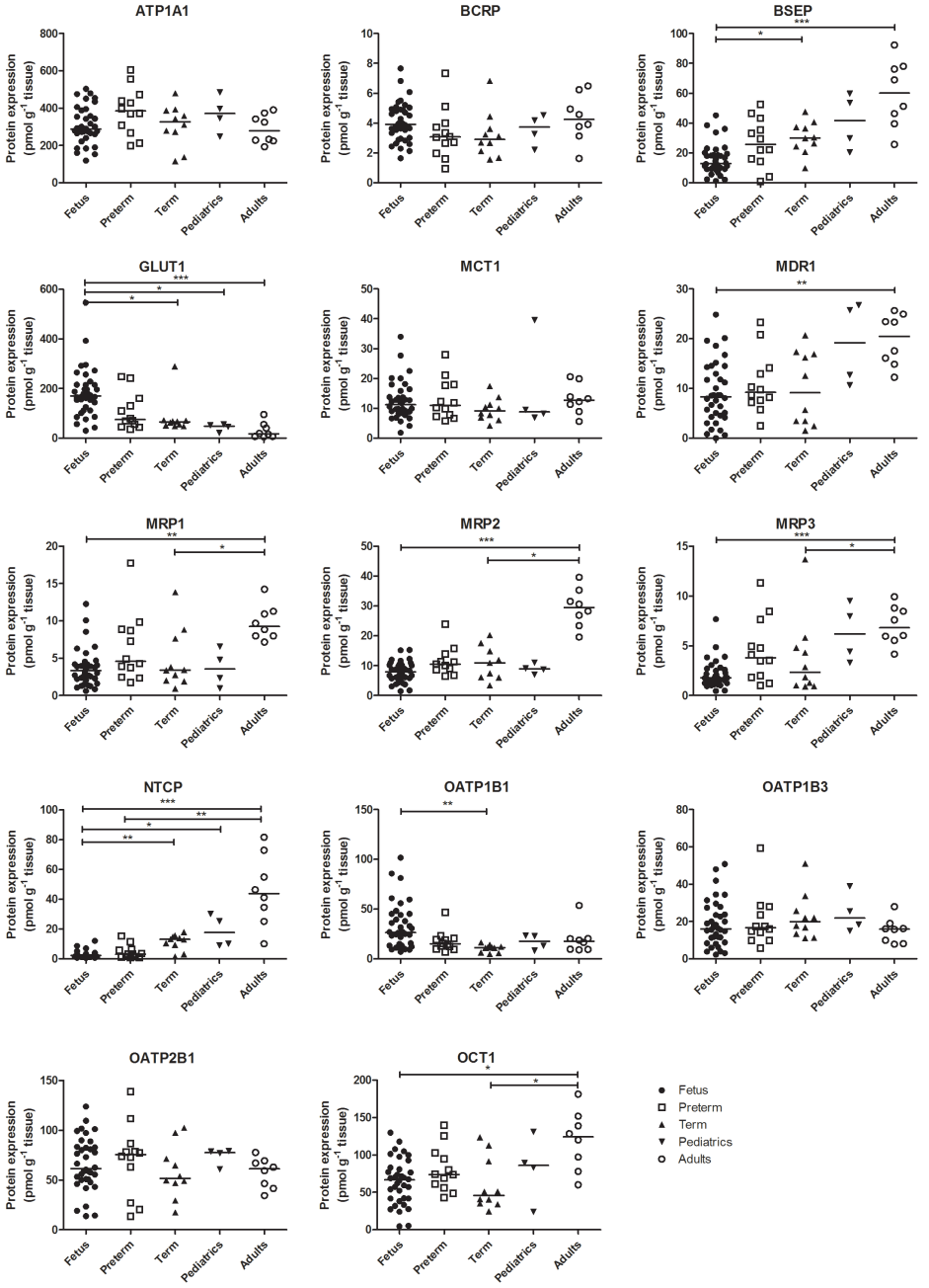
**Significant after Dunn's test (*p<0.05)*

Age-related transporter protein expression

Overall, protein expression was highly variable within age groups (Figure 2 and Supplemental table 4). More specifically, in fetal samples, BSEP and MDR1 protein expression was lower than in adult samples, and for BSEP also lower than in term newborn samples. MRP1, MRP2, MRP3 and OCT1 showed a similar developmental pattern with a lower protein expression in fetuses and newborns than in adults. NTCP levels increased over the whole age range. In contrast, GLUT1 protein levels were high in fetuses, with lower expression in term newborns, pediatrics and adults. Similarly, OATP1B1 showed high expression in the fetal age group and low expression in the term newborn age group, with stable protein levels further on. Protein expression levels of ATP1A1, BCRP, MCT1, OATP1B3 and OATP2B1 were similar in samples from all age groups.

Next, we analyzed whether GA, PNA and PMA within the fetal/newborn cohort could partly explain the observed variability (Table 2, Figure 3 and Figure 4). BCRP, BSEP and

Figure 2 Protein expression of hepatic transporters in fetuses (n=36), preterm newborns (n=12), term newborns (n=10), pediatrics (n=4) and adults (n=8).



*Significant after Dunn's test (*p<0.05, **p<0.01, ***p<0.001)

NTCP expression significantly increased with increasing GA, PNA and PMA, whereas GLUT1 and OATP1B1 decreased. For these transporters the strongest correlation was shown for with PMA. MRP2 and MRP3 were only positively correlated with PNA, and MCT1 only with PMA. When only fetal samples (postnatal age = 0) are included, the relationship between GA and transporter expression remains statistically significant for GLUT1 and OATP1B1. For the other transporters no relationship between GA, PNA or PMA and expression was found.

Correlation mRNA- and protein expression

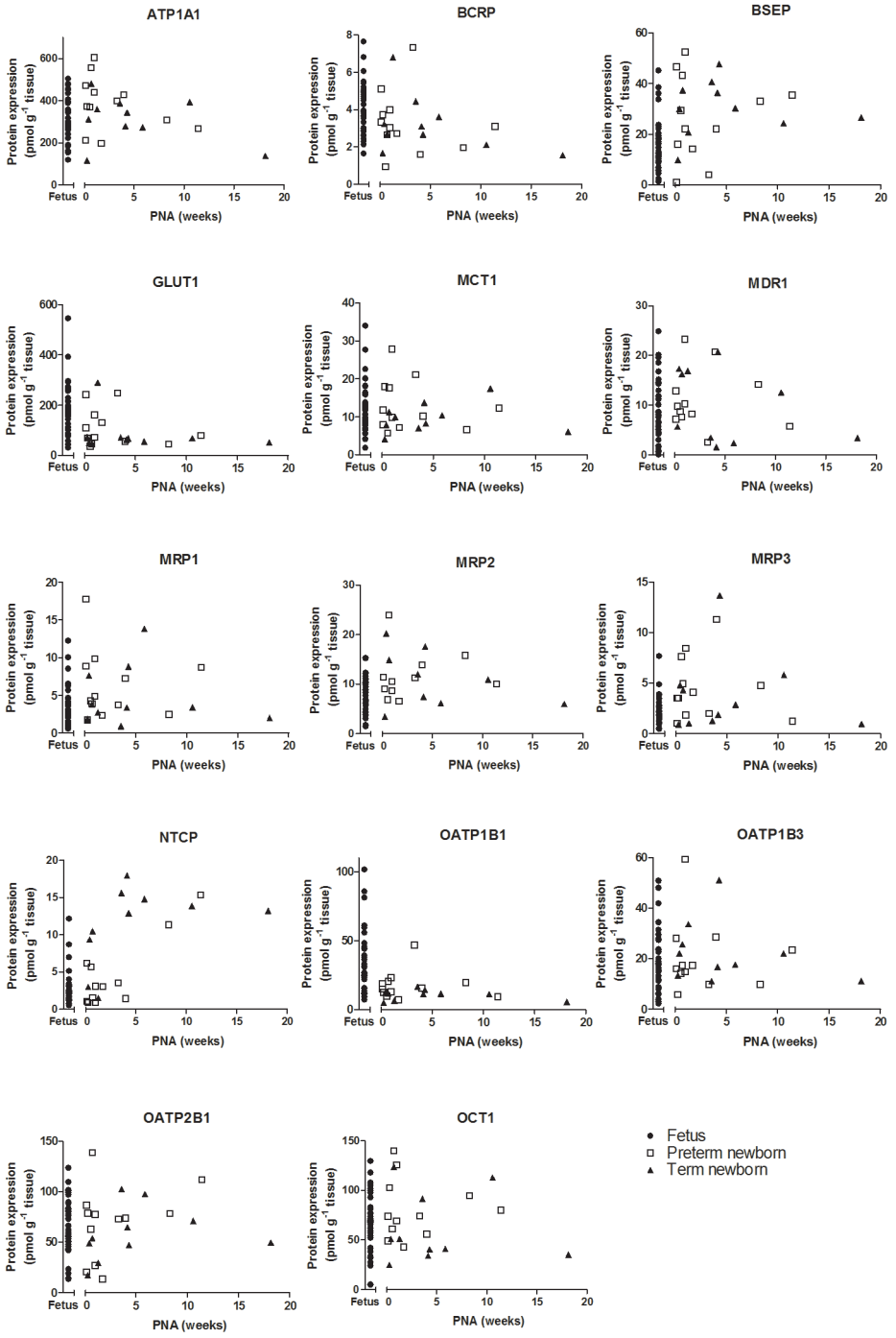
RNA-Seq data were generated from a representative subpopulation of 31 out of the 62 pediatric patients: 12 fetal (GA 29.7 weeks [15.3 – 41.3], no PNA), 8 premature newborn (GA 34.1 weeks [24.9 – 36.7], PNA 0.43 [0 – 8.29]), 7 term newborn (GA 40.0 weeks [39.7 – 41.3], PNA 3.57 [0.29 – 18.1]) and 4 pediatrics (PNA 4.13 years [1.08-7.44]). The mRNA expression levels and protein expression of ABCB11/BSEP, SLC16A1/MCT1, ABCC2/MRP2 and SLC10A1/NTCP were significantly correlated when using total TPM values of all mRNA transcripts (Supplemental Table 6). When only taking into account the mRNA transcripts actually known to be coding for protein, the correlation between mRNA expression and protein expression was lost for SLC16A1/MCT1, but appeared for ABCB1/MDR1 (Supplemental Table 6).

Table 2 Correlation of hepatic protein expression of transporters with age in fetal/newborn cohort.

Protein expression of	GA (n=58)†	GA (fetal) (n=36)‡	PNA (n=58)†	PMA (n=58)†
ATP1A1	ρ=0.120, p=0.371	ρ=0.113, p=0.513	ρ=0.145, p=0.278	ρ=0.069, p=0.605
BCRP	**ρ=-0.367, p=0.005**	ρ=-0.301, p=0.074	**ρ=-0.345, p=0.008**	**ρ=-0.421, p=0.001**
BSEP	**ρ=0.484, p<0.001**	ρ=0.230, p=0.178	**ρ=0.485, p<0.001**	**ρ=0.513, p<0.001**
GLUT1	**ρ=-0.536, p<0.001**	**ρ=-0.365, p=0.028**	**ρ=-0.512, p<0.001**	**ρ=-0.585, p<0.001**
MCT1	**ρ=-0.342, p=0.009**	ρ=-0.327, p=0.052	ρ=-0.096, p=0.473	**ρ=-0.345, p=0.008**
MDR1	ρ=-0.047, p=0.728	ρ=-0.119, p=0.489	ρ=0.064, p=0.634	ρ=-0.046, p=0.733
MRP1	ρ=0.069, p=0.608	ρ=-0.039, p=0.822	ρ=0.176, p=0.187	ρ=0.100, p=0.453
MRP2	ρ=0.202, p=0.136	ρ=0.084, p=0.625	**ρ=0.306, p=0.022**	ρ=0.214, p=0.114
MRP3	ρ=0.010, p=0.942	ρ=-0.218, p=0.202	**ρ=0.273, p=0.038**	ρ=0.032, p=0.812
NTCP	**ρ=0.502, p<0.001**	ρ=0.223, p=0.190	**ρ=0.453, p<0.001**	**ρ=0.567, p<0.001**
OATP1B1	**ρ=-0.557, p<0.001**	**ρ=-0.343, p=0.041**	**ρ=-0.481, p<0.001**	**ρ=-0.604, p<0.001**
OATP1B3	ρ=0.089, p=0.508	ρ=-0.043, p=0.804	ρ=0.090, p=0.499	ρ=0.072, p=0.589
OATP2B1	ρ=-0.135, p=0.312	ρ=-0.102, p=0.554	ρ=0.005, p=0.970	ρ=-0.092, p=0.494
OCT1	ρ=-0.206, p=0.121	ρ=-0.278, p=0.101	ρ=0.055, p=0.684	ρ=-0.175, p=0.188

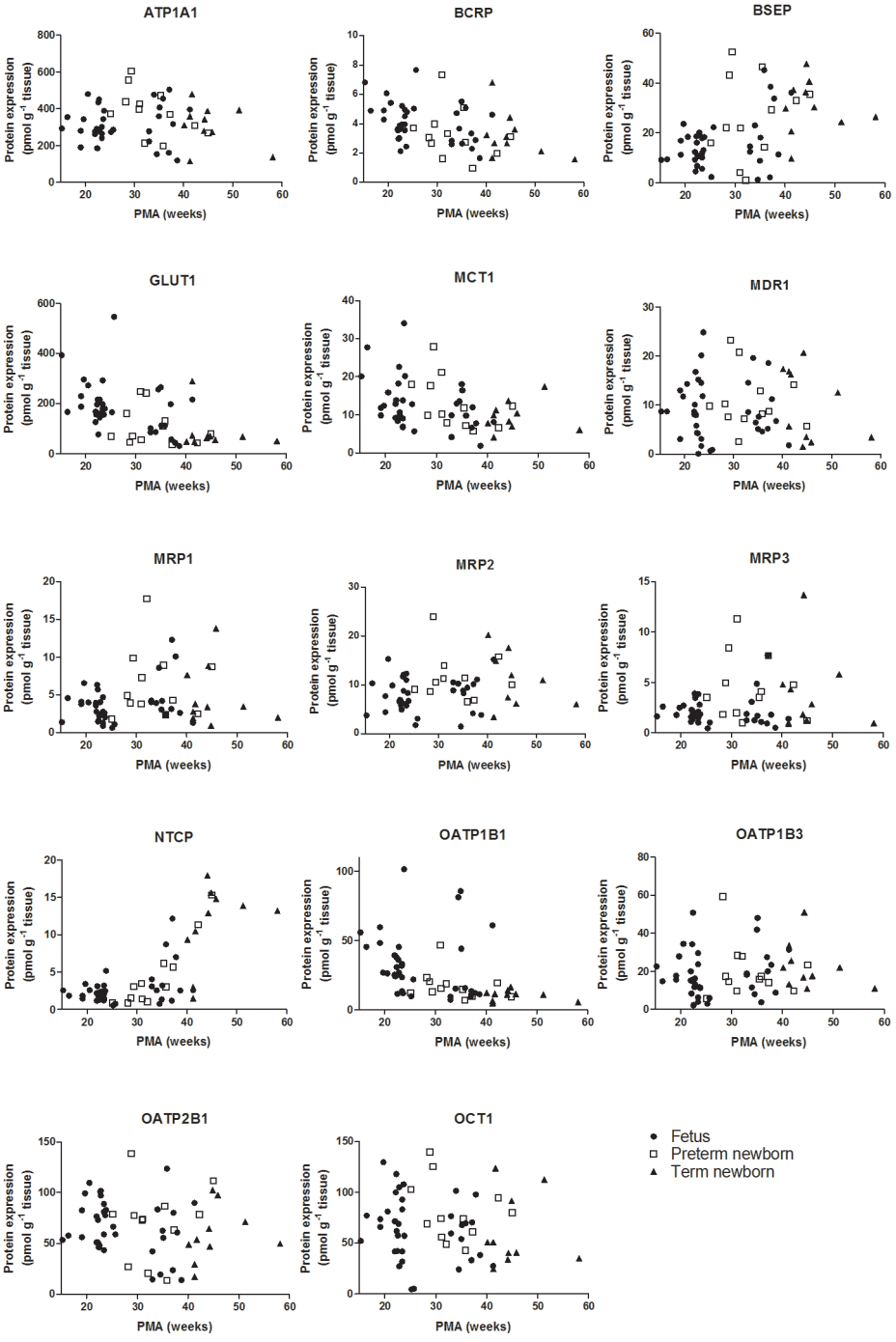
ρ= Spearman Correlation Coefficient. Bold=statistically significant (p<0.05). GA: gestational age, PNA: postnatal age, PMA: post menstrual age. †fetal/newborn cohort. ‡only fetal samples.

Figure 3 Transporter-specific postnatal maturation of hepatic protein expression in the fetal/newborn cohort (n=58).



PNA=postnatal age

Figure 4 Transporter-specific post-menstrual maturation of hepatic protein expression in the fetal/ newborn cohort (n=58).



PMA=post menstrual age

Table 3 Age-related changes in hepatic transporter expression: literature data versus our current data. See also Brouwer et al.[1]

Transporter *gene*/protein	Literature data: age vs. hepatic mRNA expression	Literature data: age vs. hepatic protein expression	Current data: age vs hepatic protein expression		
			Age groups (fetuses– preterm – term – pediatrics – adults)	PNA (between 0 – 18 weeks)	PMA (between 15.3-58.1 weeks)
ATP1A1/ ATP1A1	-	Low in neonates and increasing up to adult age.[6]†	Stable	Stable	Stable
ABCG2/BCRP	Increased levels from fetuses vs 0-4 year and >7 year [31]. Stable expression in fetal, pediatric and adult samples [32,33].	Stable from neonate to adult age [6]†. Stable in neonates and adults (n=10, western blotting) [34], and in 7-70 yr (n=56, LC-MS/MS) [22].	Stable	Decrease	Decrease
ABCB11/BSEP	3 fold lower in fetuses (n=3) than in adults (n=3) [32]. Lower in neonates than in children >7yr [31].	Stable from neonate to adult age [6]†. Detected in second trimester fetuses with immune-histochemistry [35].	Lower in fetuses than in term newborns and adults.	Increase	Increase
SLC2A1/ GLUT1	-	-	Higher in fetuses than in term newborns, pediatrics and adults.	Decrease	Decrease
SLC16A1/ MCT1	-	-	Stable	Stable	Decrease
ABCB1/MDR1	Increase in first year of life [12,33,36-38].	Low expression up to infant age and increasing to adult age [6]†. Lower in S9 liver fractions from children (7d-18yr old, n=12) than in adults [39]. Stable in a cohort from 7-70 yr (LC-MS/MS) [9].	Lower in fetuses than in adults.	Stable	Stable
ABCC1/MRP1	-	Detected in fetuses with immune-histochemistry [40].	Lower in fetuses and term newborns than in adults.	Stable	Stable
ABCC2/MRP2	Lower in fetuses, neonates and infants <1yr old than in adults [12]. Lower in fetuses compared to pediatrics from 1-17yr old [33].	Stable expression from neonate to adult age [6] and in a cohort from 7 years onward [41].	Lower in fetuses and term newborns than in adults.	Increase	Stable

Table 3 Age-related changes in hepatic transporter expression: literature data versus our current data. See also Brouwer et al.[1] *(continued)*

Transporter *gene*/protein	Literature data: age vs. hepatic mRNA expression	Literature data: age vs. hepatic protein expression	Current data: age vs hepatic protein expression		
			Age groups (fetuses– preterm – term – pediatrics – adults)	PNA (between 0 – 18 weeks)	PMA (between 15.3-58.1 weeks)
ABCC3/MRP3	Lower expression in fetuses (n=3) than in adults (n=3) [32].	Lower in infants and adolescents than in adults [6]†. Similar expression in 5 neonates and 5 adults (Western Blot) [34].	Lower in fetuses and term newborns than in adults.	Increase	Stable
SLC10A1/ NTCP	Lower in perinatal (n =6) than in 0 to 4 years (n=8), and in >7yr old (n=6) [31].	Similar in 5 neonates and 5 adults (Western Blot) [34] .	Lower in fetuses than in term newborns, pediatrics and adults. Lower in preterm newborns than in adults.	Increase	Increase
SLCO1B1/ OATP1B1	Higher in adults (n=11) than in fetuses (n=6), neonates (n=19), infants (n=7) and children (n=2) [12]. Lower in fetuses than in pediatric and adults (all n=30) [33], and lower in fetuses (n=3) than adults (n=3) [32].	Similar expression in 5 neonates and 5 adults (Western Blot) [34]. Stable in a cohort from 7-70 yr (LC-MS/MS) [9]. OATP1B1 *1/*1 showed increase in children >0 - 1 year of age [6].	High in fetuses, low in term newborns.	Decrease	Decrease
SLCO1B3/ OATP1B3	Higher in adults (n=11) than in fetuses (n=6), neonates (n=19), infants (n=7) and children (n=2) [12].	High at birth, a decline over the first months of life and an increase in preadolescent period [25]. Lower in neonates than in adults [6]†.	Stable	Stable	Stable
SLCO2B1/ OATP2B1	Lower in second trimester fetuses (n=3) than adults (n=3) [32].	Stable from neonate to adult age [6]†. Stable in a cohort from 7-70yr (LC-MS/MS) [9].	Stable	Stable	Stable
SLC22A1/ OCT1	-	Low expression from neonate up to infant age and increasing to adult age [6]†. Increase between 1-2 days (n=7) and 3-4 weeks of age (n=5) (Western Blot) [5].	Lower in fetuses and term newborns than in adults.	Stable	Stable

†Prasad et al: studied protein expression of various transporters with LC-MS/MS in the following hepatic post-mortem samples; 4 neonates (0-28 days), 19 infants (29 days – 1 year), 32 children (1-12 year), 14 adolescents (12-16 year) and 41 adults (>16 year) [6]

Genetic variants

Genotype results are presented in Supplemental Table 3. For *SLC22A1* 1222A>G, the TaqMan assay failed for two patients, presumably due to poor quality of the DNA. All patients were successfully genotyped for other SNPs. Protein expression was neither associated with the selected SNPs, nor with diplotypes of *SLCO1B1* (Supplemental Table 3), also when taking into consideration age within genotype-groups.

Cell lines

The absolute protein expression of OATP1B1, OATP1B3, OCT1, MDR1, MRP2 and BCRP was determined in the crude membrane fractions of HEK-OATP1B1, -OATP1B3, -OCT1 and MDCKII-MDR1, -MRP2, and -BCRP cells, showing good expression profiles (Supplemental Table 7).

DISCUSSION

Our study expands and presents data on human hepatic transporter protein expression in a pediatric cohort with a focus on fetal and newborn patients up to 18 weeks of postnatal age. Together with findings on gene expression and genetic variants in the same patient subcohort, this study is a comprehensive analysis of ontogeny of human hepatic drug transport in the age range where knowledge was still lacking. Below we will discuss the main findings.

Age-related changes in protein expression were transporter dependent. The results with existing data from literature are summarized in Table 3. Apart from our previous exploratory study, the only other published LC-MS/MS proteomics study we could identify included four neonates.[6] At this time, due to a lack of biological data, drug dosing in preterm and term infants is left with uncertainty regarding the level of exposure. Similarly, in pregnant women, the level of exposure to the fetus remains unknown. Our data may aid to optimize dosing of transporter substrates in these patient populations. Interestingly, when looking at age groups, most differences in transporter expression were found between the fetal and adult age groups, indicating that major changes in transporter protein expression occur in early life. For example, previously was shown that OCT1 increased from neonatal to adult age.[5,6] Our data adds that also in fetal and preterm newborns the OCT1 levels are lower than in adults. While the expression of most transporters, like OCT1, is lower in the perinatal period than at adult age, the expression of GLUT1 is significantly higher in the perinatal period. This likely reflects the physiological high need of glucose early after conception. Moreover, we did not study the transporter GLUT2, which is highly expressed in the adult liver.[20] This transporter

could be subject to age-related changes, possibly explaining our findings on GLUT1. Subsequently, OATP1B1 is also higher in the perinatal period, and is important for the hepatic uptake of hormones like estrogens.[3] Importantly, the decrease in GLUT1 and OATP1B1 may also be explained by the observed negative correlation between crude membrane yield and age. Not surprisingly, ontogeny patterns are not similar when describing transporter protein expression per membrane yield instead of per amount of tissue. However, in literature these units are used inconsistent. As transporter proteomic data is often used for PBPK modelling, coming from various sources, a correction factor should be applied when describing protein expression results per crude membrane protein in young age groups.

Both gestational age and postnatal age may impact transporter activity differently and independently. However, the combined effect, i.e. postmenstrual age, needs to be considered as well. Our data suggest that dosing of transporter substrates for BCRP, BSEP, GLUT1 and OATP1B1 is best guided by PMA in the first months of life. Using linear correlation is problematic in wide age ranges because this implies continuously increasing or decreasing expression up to adult age.[21] But as we were dealing with a limited age range (<18 weeks PNA), we considered linear correlation the most suitable to describe our data within this subpopulation.

Considerably more literature data on pediatric transporter mRNA expression is available than protein expression data.[1] However, adults studies have shown that mRNA levels do not always correlate well with transporter protein expression[22-24], which was also shown in a subpopulation of our cohort (n=31). Interestingly, the earlier found ABCB1 mRNA ontogeny pattern[12] is similar to that for the MDR1 protein in the present study, but with a much higher fold change, possibly explaining the lack of correlation. Also, post-translational changes may occur introducing differences between protein expression and protein activity. For example, a previous study found that the fraction of highly glycosylated OATP1B3 increased with age[25]. Unfortunately, because we used crude membrane fractions to measure protein expression with LC-MS/MS we could not distinguish between glycosylated and un-glycosylated transporter protein. Other techniques, e.g. Western Blot, could enable this, but this is challenging in pediatrics as much more tissue is needed.

We could not identify a relationship between protein expression and the selected genetic variants in our cohort, although these have been shown earlier to impact mRNA and/or protein expression. This finding may be explained by our low sample size, but could also partly explained by the interplay between development and genetics. For example, in a previous study *SLC22A1* 181C>T in adult samples correlated with OCT1

protein expression[26] but this was not confirmed in our cohort. OCT1 expression was low in fetuses, potentially obscuring a possible effect of genetic variants. OATP1B1 protein expression was stable within *SLCO1B1* diplotypes. In contrast, Prasad et al. showed higher protein expression in neonates versus older children/adults with the *SLCO1B1* *1A/*1A haplotype.[6] Moreover, our group previously showed that the *SLC22A1* genotype is related to tramadol disposition in preterm infants, similar to adults.[10] This suggests that, although protein levels are low, the *SLC22A1* genotype can result in significant differences in protein activity in neonates. Thus, although we did not find a correlation between interrogated SNPs and protein expression, it remains important to include genotype when analyzing developmental patterns.

Some potential limitations of our study should be addressed. First, our results show high inter-individual variation in transporter protein expression, which in part remained unexplained by age, gender and genotype. It is well possible that inflammation[27], disease, nutrition and drugs influenced transporter expression in our cohort. Healthy infants do not require medications like ill newborns do, thus our cohort represents the relevant population for our intended purpose. The relative impact of these factors, however, deserves further study. Also, samples were snap-frozen at -80°C for later research use at the time of autopsy within 24 hours after death, which might have introduced differences in quality of tissue. These limitations warrant careful interpretation of our data.

Nevertheless, our data help improve our understanding of drugs and endogenous processes in human populations of different ages. Moreover, our data could be integrated in PBPK modeling, which might improve prediction of pediatric drug clearance. Because differences might exist between protein expression and protein activity, future perspectives will be to validate these models with clinical data from transporter substrates. Previously, we have shown the value of determining absolute transporter protein expressions in transfected cell lines for application in PBPK modelling: *in vivo* hepatic disposition of rosuvastatin was predicted by scaling from individually transfected cell lines by correcting for absolute transporter protein expression levels.[28] In the current study we therefore determined the absolute expression levels of the transporter protein in selected relevant cell lines, frequently applied in *in vitro* drug metabolism PK studies. Hence, the obtained results can be incorporated into PBPK modeling to extrapolate existing adult PK data to pediatric PK data[29,30], or used as appropriate scaling factors to scale between in vitro cell lines and human hepatic expression in adults or pediatric patients.

CONCLUSIONS

In conclusion, we observed various patterns in the maturation of protein expression of a number of hepatic transporter proteins in children up to four months. This strongly suggests that disposition of drugs and endogenous transporter substrates is subject to age-related changes and impacts the efficacy and safety of drugs in the first months of life. Postmenstrual age may present the most robust method to incorporate age-related variation in transporter protein expression in dosing guidelines. mRNA expression as surrogate marker of transporter activity should be carefully interpreted as correlation with protein expression is mostly lacking. Moreover, adult pharmacogenetic data cannot be directly extrapolated to neonates and young infants. Further study is needed to delineate the effect on *in vivo* drug disposition and effect.

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SUPPLEMENTAL INFORMATION

Supplemental Table 1 Clinical diagnoses pediatric patients

Clinical diagnosis	Number of patients
Congenital malformations (cardiac, otolaryngeal, chromosomal, abdominal, unknown)	32
Intrauterine death	5
Hydrops fetalis	2
Viral/bacterial infections	7
Cardiac failure	5
Necrotizing enterocolitis	3
Hemangioendothelioma	1
Sudden infant death syndrome	2
Intracranial bleeding	1
Meconium aspiration	1
Pulmonary hypertension	1
Neurologic abnormality	1
Hernia incarcerate	1

Supplemental Table 2 Multiple reaction monitoring (MRM) transitions of the used peptides and the corresponding internal standards (AQUA)

Name	Labelled	Peptide sequence[a]	Molecular weight	Q1	Q3-1	Q3-2	Q3-3	Q3-4
ATP1A1	unlabeled	AAVPDAVGK	827.0	414.2	586.3	685.4	242.1	
	AQUA	AAVPDA***V***GK	833.0	417.5	592.3			
BCRP	unlabeled	SSLLDVLAAR	1.044.2	522.8	644.3	757.5	529.4	
	AQUA	SSLLDV***L***AAR	1.060.2	526.3	651.3			
BSEP	unlabeled	STALQLIQR	1.029.2	515.3	657.4	841.6	529.4	
	AQUA	STALQ***L***IQR	1.045.2	518.8	664.3			
GLUT1	unlabeled	VTILELFR	990.2	495.8	790.5	677.4	201.2	
	AQUA	VTILEL***F***R	1.000.2	500.8	800.5			
MCT1	unlabeled	SITVFFK	841.0	421.2	173.2	641.3	201.1	
	AQUA	SITVF***F***K	851.0	426.2	651.3			
MDR1	unlabeled	NTTGALTTR	934.0	467.7	719.4	216.1	618.4	
	AQUA	NTTGA***L***TTR	950.0	471.2	726.5			
MRP2	unlabeled	VLGPNGLLK	910.1	455.8	698.5	185.3	213.3	
	AQUA	VLGPNGL***L***K	926.1	459.2	705.4			
MRP3	unlabeled	ALVITNSVK	944.1	472.8	760.4	661.4	548.4	
	AQUA	ALVITNS***V***K	950.1	475.8	766.5			
NTCP	unlabeled	GIYDGDLK	880.44	440.7	710.3	143.2	171.2	
	AQUA	GIYDGD***L***K	896.44	444.2	717.3			
OATP1B1	unlabeled	LNTVGIAK	815.0	408.2	399.4	588.3	228.2	702.3
	AQUA	LNTVG***I***AK	831.0	411.7	402.9			
OATP1B3	unlabeled	IYNSVFFGR	1101.3	551.8	826.5	249.1	526.2	
	AQUA	IYNSVF***F***GR	1111.6	556.8	836.4			
OATP2B1	unlabeled	SSISTVEK	849.9	425.7	563.3	676.3	175.1	
	AQUA	SSIST***V***EK	855.9	428.7	569.3			
OCT1	unlabeled	LPPADLK	752.9	377.2	543.3	183.3	260.3	
	AQUA	LPPAD***L***K	768.9	380.7	550.4			

*[a] AQUA: Amino acid presented in **italic bold** is labelled with ^{13}C and ^{15}N.*

Supplemental Table 3 Overview selection of SNPs known to influence mRNA- or protein expression. Number of livers from carriers of various SNPs present in the studied cohort. *Kruskal-Wallis test

Gene	Gene SNP ID	Variant	SNP class	Genotype			Distribution protein expression across genotype groups*
				Wildtype (n=)	Hetero-zygous (n=)	Homo-zygous (n=)	
ABCG2	rs2231142	421C>A	Missense	50	10	0	p=0.132
ABCB1	rs1045642	3435C>T	Synonymous	15	27	18	p=0.883
ABCC1	rs45511401	16079375G>T	Missense	56	4	0	p=0.155
ABCC2	rs2273697	1249G>A	Missense	35	19	6	p=0.197
ABCC3	rs4793665	-211C>T	5 Flanking	15	22	23	p=0.792
SLCO1B1	rs4149056	521T>C	Missense	44	16	0	p=0.132
	rs2306283	388A>G	Missense	10	33	17	p= 0.821
	rs11045819	463C>A	Missense	45	12	3	p= 0.324
SLCO1B3	rs4149117	334T>G	Missense	1	19	40	p=0.459
	rs7311358	699G>A	Missense	1	19	40	p=0.459
SLCO2B1	rs2306168	1457C>T	Missense	54	4	1	p=0.132
	rs12422149	935G>A	Missense	49	10	1	p=0.682
SLC22A1	rs12208357	181C>T	Missense	55	5	0	p=0.841
	rs628031	1222A>G	Missense	9	25	24	p=0.206

Supplemental Table 4 hepatic protein expression of transporters in each age group. Data is presented as median (range).

	Transporter	All n=70	Fetuses n=36	Preterm newborns n=12	Term newborns n=10	Pediatrics n=4	Adults n=8	Expression difference across age groups ‡
Hepatic protein expression (pmol g^{-1} tissue)	ATP1A1	309.8 (115.7-604.3)	288.2 (119.3-503.6)	384.9 (197.6-604.3)	327.3 (115.7-479.6)	371.5 (247.3-484.1)	279.3 (193.8-391.2)	p=0.236
	BCRP	3.7 (0.9-7.7)	3.9 (1.7-7.7)	3.1 (0.9-7.3)	2.9 (1.6-6.8)	3.7 (2.2-4.5)	4.2 (1.6-6.5)	p=0.120
	BSEP	20.5 (0.9-92.4)	12.8 (1.3-45.2)	25.7 (0.9-52.4)	30.1 (9.8-47.7)	41.7 (20.4-59.7)	60.1 (25.7-92.4)	p<0.001
	GLUT1	105.5 (6.3-546.2)	170.4 (30.3-546.2)	75.2 (35.2-247.9)	65.0 (48.1-290.0)	48.8 (21.4-55.3)	17.8 (6.3-95.0)	p<0.001
	MCT1	10.5 (1.9-39.5)	11.2 (1.9-34.0)	11.0 (5.8-27.9)	9.2 (4.2-17.5)	8.9 (6.9-39.5)	12.8 (5.7-20.6)	p=0.598
	MDR1	10.2 (0.04-26.7)	8.3 (0.04-24.9)	9.2 (2.5-23.3)	9.1 (1.5-20.7)	19.2 (10.6-26.7)	20.5 (12.3-25.7)	p=0.002
	MRP1	3.9 (0.6-17.7)	3.4 (0.6-12.3)	4.6 (1.8-17.7)	3.4 (0.9-13.8)	3.6 (1.0-6.6)	9.3 (7.2-14.2)	p=0.001
	MRP2	9.3 (1.5-39.6)	8.0 (1.5-15.3)	10.5 (6.5-23.9)	10.9 (3.4-20.23)	8.9 (7.0-11.0)	29.5 (19.6-39.6)	p<0.001
	MRP3	2.4 (0.5-13.7)	1.8 (0.5-7.7)	3.8 (1-11.3)	2.4 (0.9-13.7)	6.2 (3.3-9.5)	6.8 (4.2-9.9)	p<0.001
	NTCP	3.1 (0.6-81.6)	2.3 (0.6-12.2)	3.1 (0.9-15.3)	13.1 (1.5-18.0)	17.7 (8.8-30.1)	43.8 (10.2-81.6)	p<0.001
	OATP1B1	17.7 (4.9-101.7)	26.6 (7.1-101.7)	15.0 (7.0-46.8)	11.3 (4.9-16.5)	17.9 (8.2-23.2)	17.7 (9.1-53.7)	p=0.001
	OATP1B3	16.6 (2.3-59.3)	16.0 (2.3-50.9)	16.7 (5.8-59.3)	19.9 (11.1-51.1)	21.9 (15.1-38.9)	16.0 (7.9-28.1)	p=0.566
	OATP2B1	63.9 (13.5-138.7)	61.6 (13.8-123.8)	75.5 (13.5-138.7)	51.9 (17.5-102.6)	77.7 (60.8-79.1)	61.3 (34.5-77.8)	p=0.503
	OCT1	70.0 (4.6-181.4)	66.7 (4.6-129.7)	73.9 (42.7-139.7)	45.9 (24.6-123.8)	86.1 (23.9-131.2)	124.3 (60.1-181.4)	p=0.010

GA=gestational age, PNA=postnatal age, NA=not available. ‡Kruskal-Wallis test.

Supplemental Table 5 Hepatic protein expression of transporters in males and females. Data is presented as median (range).* Mann Whitney U test.

Transporter	Hepatic expression (pmol/g tissue)		Distribution over groups*
	Male (n=35)	Female (n=27)	
ATP1A1	292.5 (115.7-604.9)	342.9 (137.6-471.9)	p=0.848
BCRP	3.5 (1.6-6.8)	4.0 (0.9-7.7)	p=0.122
BSEP	18.0 (0.9-59.7)	20.4 (4.0-47.7)	p=0.324
GLUT1	112.0 (21.4-393.0)	144.3 (35.2-546.2)	p=0.804
MCT1	12.0 (1.9-39.5)	9.8 (5.7-34.0)	p=0.189
MDR1	8.1 (0.7-24.9)	11.2 (0.1-26.7)	p=0.456
MRP1	3.8 (0.6-17.7)	3.8 (0.9-13.8)	p=0.609
MRP2	8.8 (1.5-23.9)	8.8 (3.1-20.2)	p=0.633
MRP3	1.8 (0.5-11.3)	2.5 (1.0-13.7)	p=0.138
NTCP	2.6 (0.6-30.1)	3.2 (0.8-15.6)	p=0.284
OATP1B1	20.4 (4.9-101.7)	14.7 (5.7-85.8)	p=0.624
OATP1B3	18.2 (2.3-59.3)	16.4 (3.9-51.1)	p=0.537
OATP2B1	64.6 (13.8-138.7)	72.9 (13.5-123.8)	p=0.189
OCT1	67.6 (4.6-139.7)	69.6 (5.2-129.7)	p=0.771

Supplemental Table 6 Correlation of mRNA- and protein expression of hepatic transporters. mRNA expression is presented as median (range). ρ= Spearman Correlation Coefficient. Bold=statistically significant (p<0.05)

Transporter	mRNA expression all transcripts (TPM)	Correlation protein expression and all mRNA transcripts	mRNA expression coding transcripts (TPM)	Correlation protein expression and coding mRNA transcripts
ATP1A1	20.9 (4.4-168.5)	ρ=-0.123, p=0.510	12.1 (0.6-105.7)	ρ=0.144, p=0.441
ABCG2/BCRP	1.8 (0.0-7.7)	ρ=-0.080, p=0.670	1.8 (0.0-7.7)	ρ=-0.080, p=0.670
ABCB11/BSEP	8.3 (0.0-41.8)	**ρ=0.533, p=0.002**	7.5 (0.0-27.3)	**ρ=0.525, p=0.002**
SLC2A1/GLUT1	9.0 (0.0-125.7)	ρ=0.293, p=0.110	6.2 (0.0-92.9)	ρ=0.313, p=0.087
SLC16A1/MCT1	9.1 (0.0-77.0)	**ρ=0.517, p=0.003**	4.3 (0.0-19.7)	ρ=0.342, p=0.060
ABCB1/MDR1	1.9 (0.0-8.6)	ρ=0.150, p=0.419	0.9 (0.0-5.0)	**ρ=0.394, p=0.028**
ABCC1/MRP1	2.6 (0.0-17.5)	ρ=0.118, p=0.527	0.6 (0.0-6.2)	ρ=0.104, p=0.577
ABCC2/MRP2	15.9 (0.4-70.6)	**ρ=0.467, p=0.011**	14.7 (0.4-61.8)	**ρ=0.512, p=0.005**
ABCC3/MRP3	11.1 (0.0-128.7)	ρ=0.096, p=0.606	1.4 (0.0-5.6)	ρ=0.260, p=0.158
SLC10A1/NTCP	1.4 (0.0-26.0)	**ρ=0.584, p=0.001**	1.4 (0.0-26.0)	**ρ=0.584, p=0.001**
SLCO1B1/OATP1B1	26.1 (0.0-69.4)	ρ=0.182, p=0.328	26.1 (0.0-69.4)	ρ=0.182, p=0.328
SLCO1B3/OATP1B3	10.6 (0.0-56.8)	ρ=-0.093, p=0.620	9.2 (0.0-49.6)	ρ=-0.114, p=0.541
SLCO2B1/OATP2B1	24.7 (0.4-84.0)	ρ=0.296, p=0.106	17.6 (0.3-72.6)	ρ=0.313, p=0.087
SLC22A1/OCT1	2.4 (0.0-122.1)	ρ=-0.032, p=0.865	1.4 (0.0-57.6)	ρ=-0.090, p=0.629

Supplemental Table 7 Absolute transporter expression in selected cell-lines

	Absolute transporter expression (fmol/106 cells)	
	Mean	SD
HEK-OATP1B1	143.5	8.8
HEK-OATP1B3	400.2	31.2
HEK-OCT1	667.0	174.2
MDCKII-MDR1	832.4	42.9
MDCKII-MRP2	54.8	1.9
MDCKII-BCRP	301.6	4.5