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# 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 BCRP, BSEP, GLUT1, MDR1, MRP1, MRP2, MRP3, NTCP, OATP1B1 and OCT1, 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 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.

*Abbreviations:* BCRP, breast cancer resistant protein; BSEP, bile salt efflux pump; GA, gestational age; GLUT1, glucose transporter 1; MCT1, monocarboxylate transporter 1; MDR, multidrug-resistance protein; MRP, multidrug-resistance like protein; NTCP, Na+-taurocholate cotransporting polypeptide; OATP, organic anion transporter polypeptide; OCT1, organic cation transporter 1; PBPK, physiologically-based pharmacokinetic; PNA, postnatal age; PMA, postmenstrual age; SNP, single nucleotide polymorphism; UGT, UDP-glucuronosyltransferase

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## 1. 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 (Brouwer et al., 2015).

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 (Hsieh et al., 2014). Many of these drugs are substrates for transporters (Mooij et al., 2016a), and the expression and activity of certain transporters are known to be subject to age-related changes (Brouwer et al., 2015). 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 (Tzvetkov et al., 2013). Data suggest lower protein expression of hepatic OCT1 in younger age groups (Hahn et al., 2017; Prasad et al., 2016), 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 (Brouwer et al., 2015; Mooij et al., 2016a).

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 GA and PNA. Both GA and birth are important determinants of postnatal gene expression of drug metabolizing enzymes (Hines, 2008). 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 (Mooij et al., 2016b). 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 (Prasad et al., 2016). Data on gene expression of transporters in the younger ages are richer (Brouwer et al., 2015; Mooij et al., 2016b), 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 (Prasad et al., 2014). 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 (Matic et al., 2016). 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.

## 2. Material and methods

### 2.1. 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 h after death and snap-frozen at −80 °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.

### 2.2. 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 (Mooij et al., 2016b), but was added in this expanded study because of their clinical relevance. We also selected ATP1A1, which is often used as a housekeeping protein (Prasad et al., 2016).

### 2.3. 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 (van de Steeg et al., 2013), 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 $20 \times 10^6$ 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.

**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.

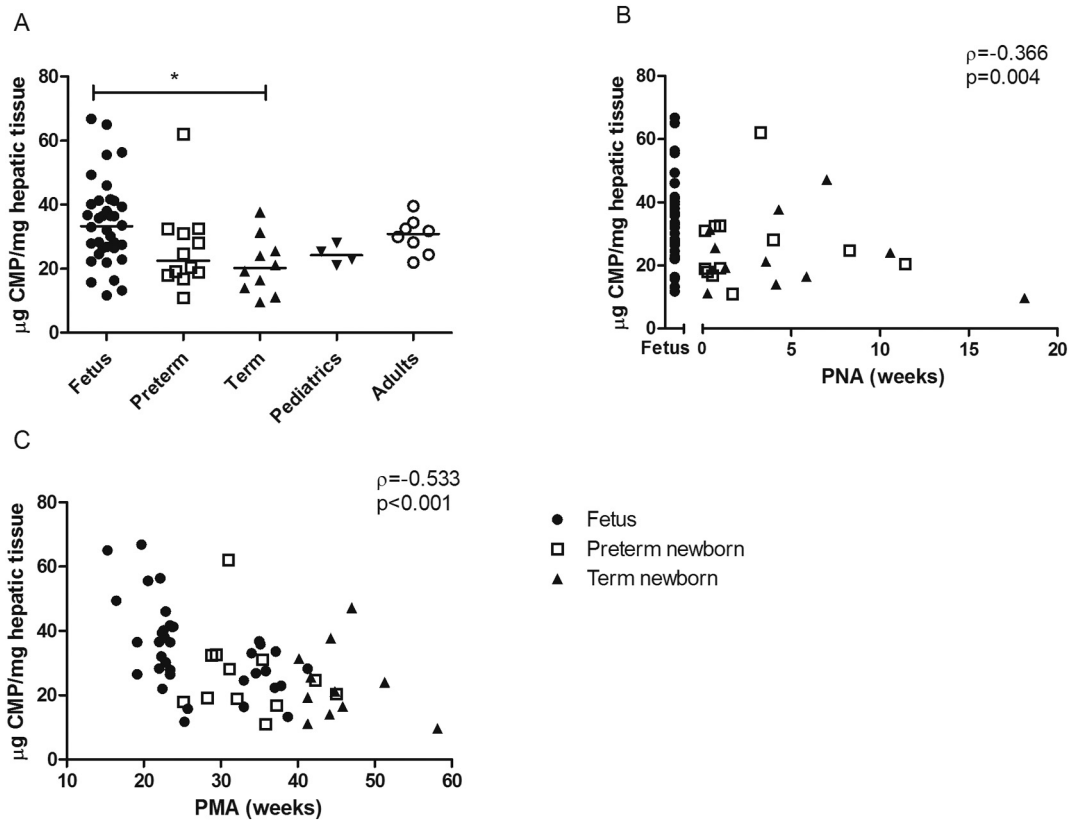



**Fig. 1.** Crude membrane protein (CMP) yield per amount of hepatic tissue, presented for various age groups (A), and, for the fetal/newborn cohort, for postnatal age (PNA) (B) and postmenstrual age (PMA) (C). ρ = Spearman's rho. *Significant after Dunn's test (*$p < 0.05$).

Samples were diluted with 2 volumes of 90% methanol. The proteins were subsequently reduced with 0.01 M dithiothreitol at 37 °C for 60 min and alkylated with 0.04 M iodoacetamide for 20 min 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 h 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 (van de Steeg et al., 2013). 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.

### 2.4. 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. (2014). 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 (Langmead and Salzberg, 2012) 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 (Li and Dewey, 2011). The counts of RNA-Seq fragments were used to indicate the amount of identified mRNA transcripts, presented in transcripts per million (TPM) (Li and Dewey, 2011).

For each transporter we calculated the total TPM values of all mRNA

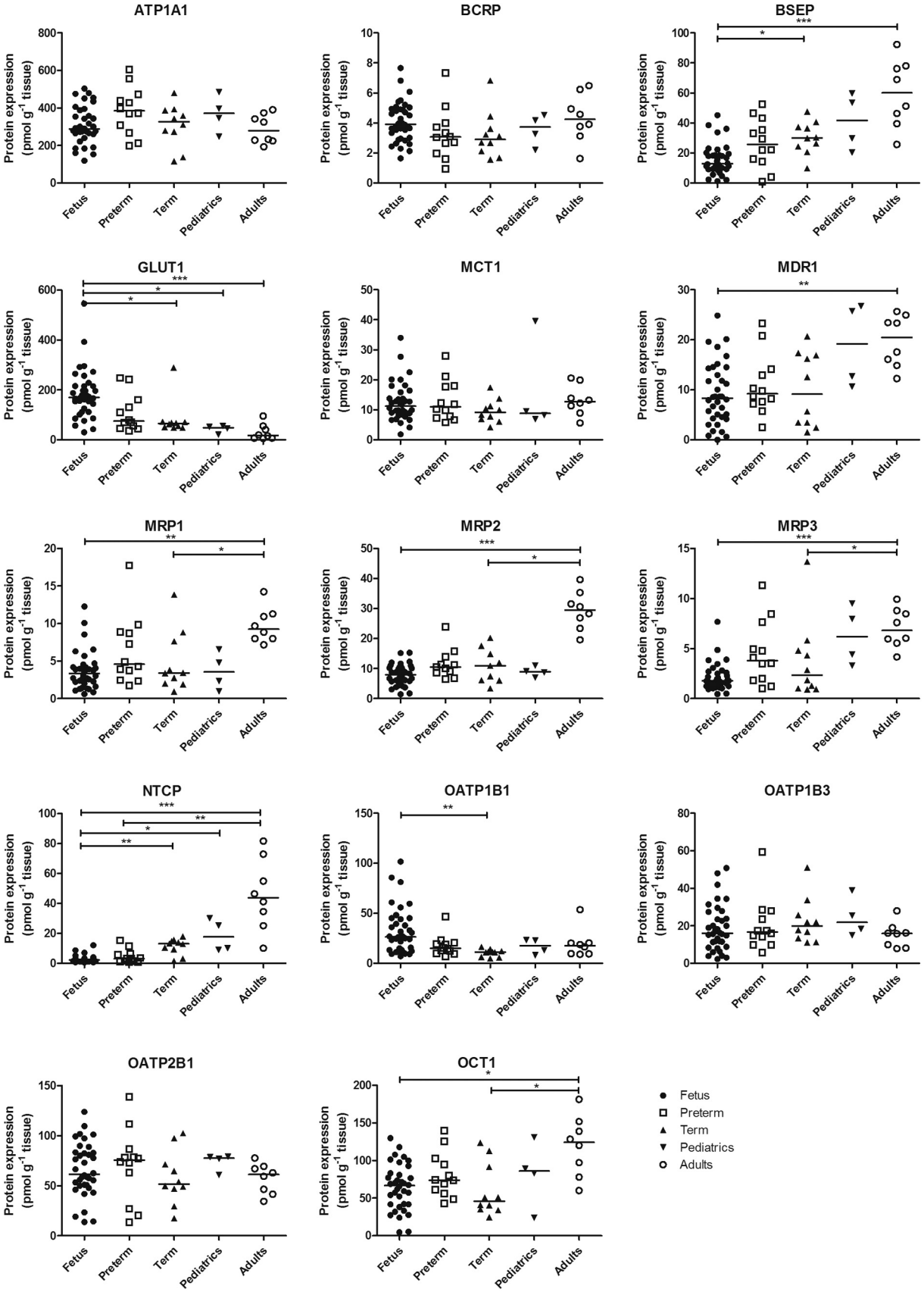



**Fig. 2.** Protein expression of hepatic transporters in fetuses ($n$ = 36), preterm newborns ($n$ = 12), term newborns ($n$ = 10), pediatrics ($n$ = 4) and adults ($n$ = 8). *Significant after Dunn's test (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

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

| Protein expression of | GA ($n$ = 58)[a] | GA (fetal) ($n$ = 36)[b] | PNA ($n$ = 58)[a] | PMA ($n$ = 58)[a] |
|---|---|---|---|---|
| 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.
[a] Fetal/newborn cohort.
[b] Only fetal samples.

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.

### 2.5. 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. 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 haplotypes of *SLCO1B1* on protein expression was shown (Prasad et al., 2014), 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 1× TE Buffer to a 10 ng $\mu L^{-1}$ 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).

### 2.6. 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 (van de Steeg et al., 2013, 2015). 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) (Evers et al., 1998; Jonker et al., 2002, 1999).

### 2.7. 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).

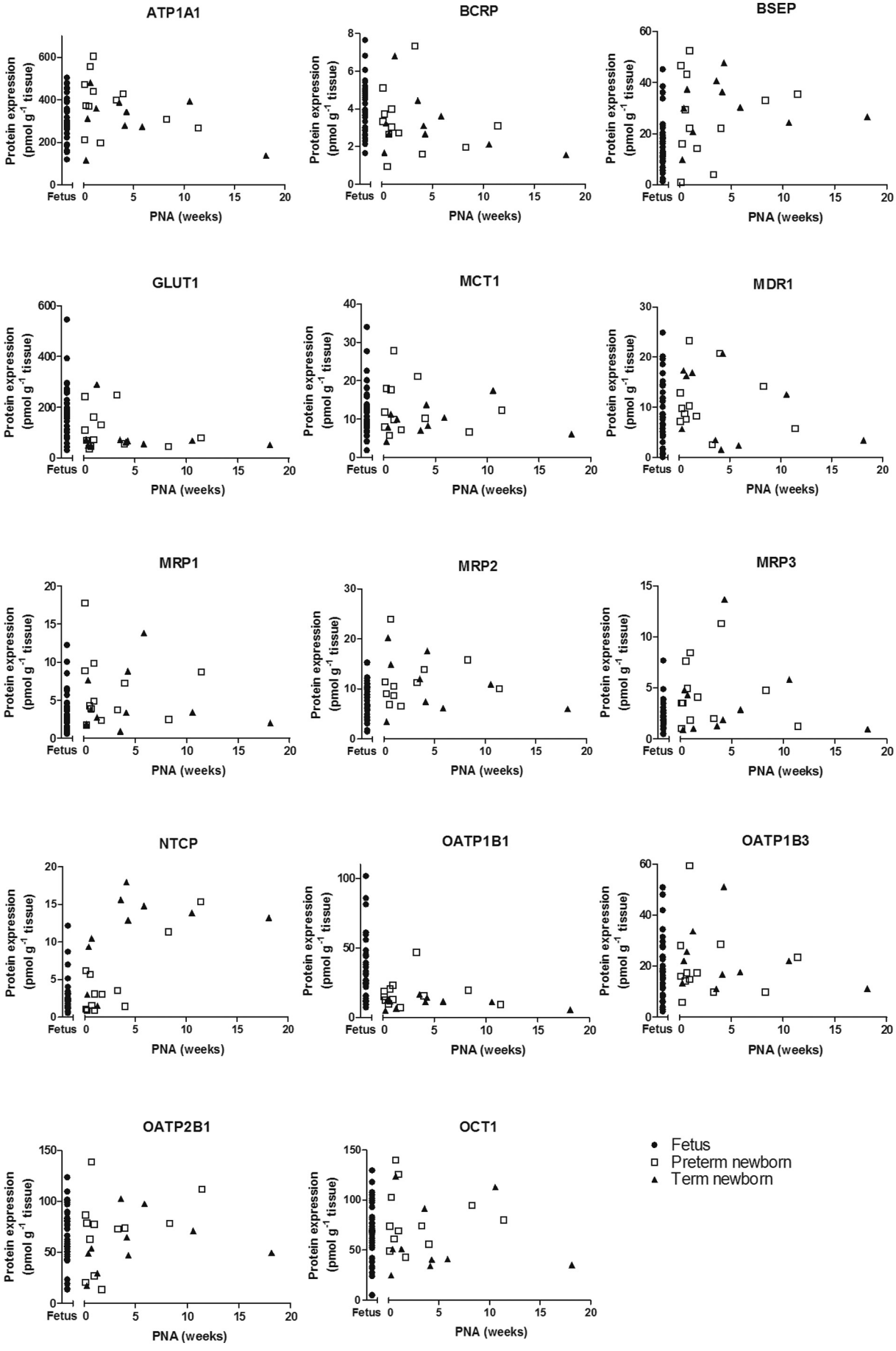
## 3. Results

### 3.1. Descriptive results

In total 71 hepatic tissue samples were available for the study, including 25 samples of our pilot study (Mooij et al., 2016b). 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.

### 3.2. 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 (Fig. 1A). Moreover, it was negatively correlated with PNA and PMA in




*(caption on next page)*

**Fig. 3.** Transporter-specific postnatal maturation of hepatic protein expression in the fetal/newborn cohort ($n$ = 58). PNA = postnatal age.

the fetal/newborn cohort (Fig. 1B and C, respectively).

### 3.3. Age-related transporter protein expression

Overall, protein expression was highly variable within age groups (Fig. 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 term 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, Figs. 3 and 4). BSEP and NTCP expression significantly increased with increasing GA, PNA and PMA, whereas BCRP, 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 GA and 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.

### 3.4. 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).

### 3.5. 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 haplotypes of *SLCO1B1* (Supplemental Table 3), also when taking into consideration age within genotype-groups.

### 3.6. 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).

## 4. 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 (Prasad et al., 2016). 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 (Hahn et al., 2017; Prasad et al., 2016). 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. However, we did not study the transporter GLUT2, which is highly expressed in the adult liver (Karim et al., 2012). 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 (Mooij et al., 2016a). 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 modeling, 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 (Leeder and Meibohm, 2016). 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 (Brouwer et al., 2015). However, adults studies have shown that mRNA levels do not always correlate well with transporter protein expression (Maier et al., 2009; Prasad et al., 2013; Ulvestad et al., 2013), which was also shown in a subpopulation of our cohort ($n$ = 31). Interestingly, the earlier found ABCB1 mRNA ontogeny pattern (Mooij et al., 2014) 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

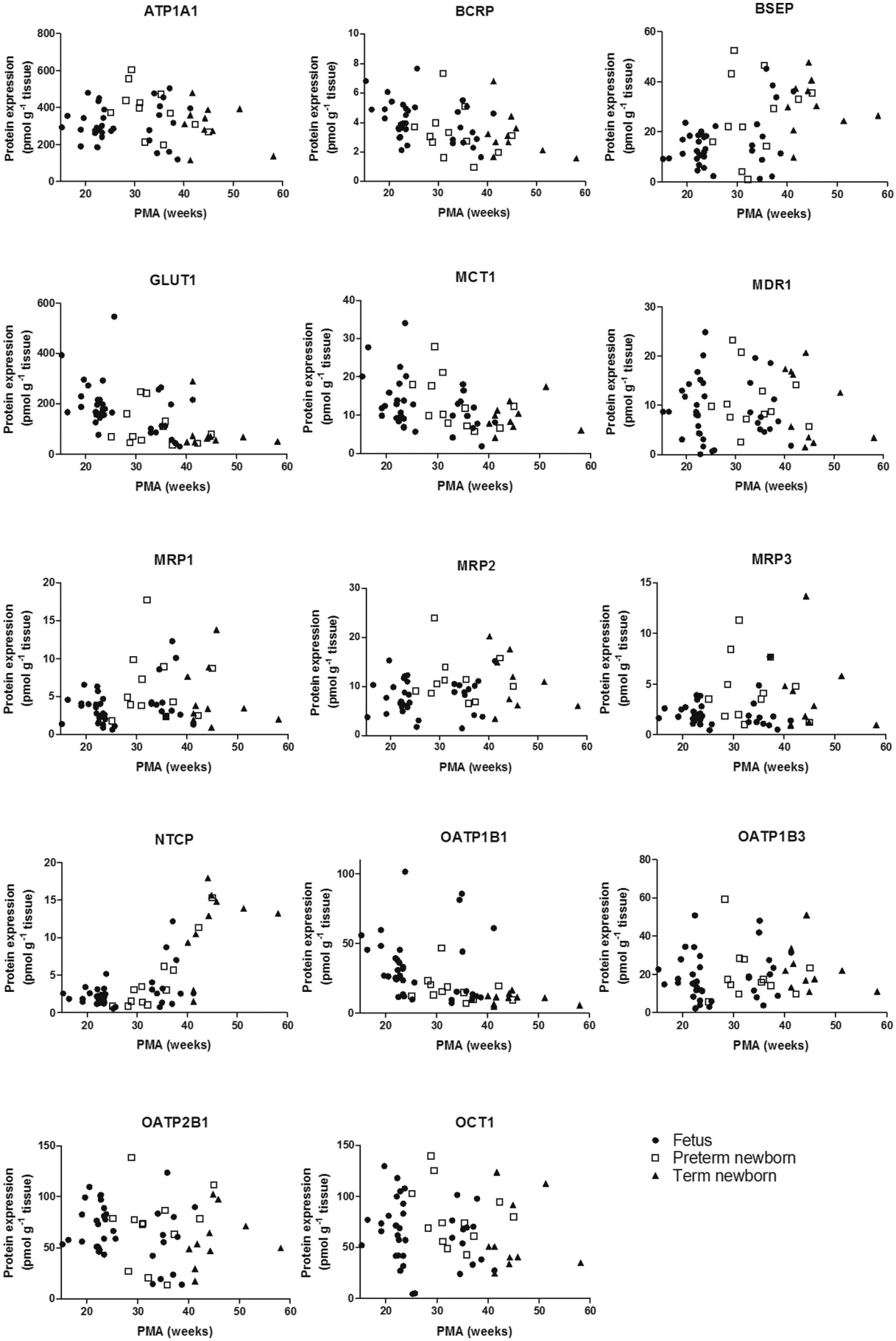



**Fig. 4.** Transporter-specific post-menstrual maturation of hepatic protein expression in the fetal/newborn cohort ($n$ = 58). PMA = post-menstrual age.

**Table 3**
Age-related changes in hepatic transporter expression: literature data versus our current data. See also Brouwer et al. (2015).

| 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 and 18 weeks) | PMA (between 15.3 and 58.1 weeks) |
| *ATP1A1*/ATP1A1 | – | Low in neonates and increasing up to adult age.(Prasad et al., 2016)[a] | Stable | Stable | Stable |
| *ABCG2*/BCRP | Increased levels from fetuses vs 0–4 year and > 7 year (Klaassen and Aleksunes, 2010). Stable expression in fetal, pediatric and adult samples (Burgess et al., 2015; Sharma et al., 2013). | Stable from neonate to adult age (Prasad et al., 2016)[a]. Stable in neonates and adults ($n$ = 10, Western blotting) (Yanni et al., 2011), and in 7–70 yr ($n$ = 56, LC-MS/MS) (Prasad et al., 2013). | Stable | Decrease | Decrease |
| *ABCB11*/BSEP | 3 fold lower in fetuses ($n$ = 3) than in adults ($n$ = 3) (Sharma et al., 2013). Lower in neonates than in children > 7 yr (Klaassen and Aleksunes, 2010). | Stable from neonate to adult age (Prasad et al., 2016)[a]. Detected in second trimester fetuses with immune-histochemistry (Chen et al., 2005). | 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 (Burgess et al., 2015; Fakhoury et al., 2009; Miki et al., 2005; Mooij et al., 2014; van Kalken et al., 1992). | Low expression up to infant age and increasing to adult age (Prasad et al., 2016)[a]. Lower in S9 liver fractions from children (7 d–18 yr old, $n$ = 12) than in adults (Abanda et al., 2017). Stable in a cohort from 7 to 70 yr (LC-MS/MS) (Prasad et al., 2014). | Lower in fetuses than in adults. | Stable | Stable |
| *ABCC1*/MRP1 | – | Detected in fetuses with immune-histochemistry (Konieczna et al., 2011). | Lower in fetuses and term newborns than in adults. | Stable | Stable |
| *ABCC2*/MRP2 | Lower in fetuses, neonates and infants < 1 yr old than in adults (Mooij et al., 2014). Lower in fetuses compared to pediatrics from 1 to 17 yr old (Burgess et al., 2015). | Stable expression from neonate to adult age (Prasad et al., 2016) and in a cohort from 7 years onward (Deo et al., 2012). | Lower in fetuses and term newborns than in adults. | Increase | Stable |
| *ABCC3*/MRP3 | Lower expression in fetuses ($n$ = 3) than in adults ($n$ = 3) (Sharma et al., 2013). | Lower in infants and adolescents than in adults (Prasad et al., 2016)[a]. Similar expression in 5 neonates and 5 adults (Western blot) (Yanni et al., 2011). | 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 > 7 yr old ($n$ = 6) (Klaassen and Aleksunes, 2010). | Similar in 5 neonates and 5 adults (Western blot) (Yanni et al., 2011). | 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) (Mooij et al., 2014). Lower in fetuses than in pediatric and adults (all $n$ = 30) (Burgess et al., 2015), and lower in fetuses ($n$ = 3) than adults ($n$ = 3) (Sharma et al., 2013). | Similar expression in 5 neonates and 5 adults (Western blot) (Yanni et al., 2011). Stable in a cohort from 7 to 70 yr (LC-MS/MS) (Prasad et al., 2014). OATP1B1 *1/*1 showed increase in children > 0–1 year of age (Prasad et al., 2016). | Higher in fetuses than 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) (Mooij et al., 2014). | High at birth, a decline over the first months of life and an increase in preadolescent period (Thomson et al., 2016). Lower in neonates than in adults (Prasad et al., 2016)[a]. | Stable | Stable | Stable |
| *SLCO2B1*/ OATP2B1 | Lower in second trimester fetuses ($n$ = 3) than adults ($n$ = 3) (Sharma et al., 2013). | Stable from neonate to adult age (Prasad et al., 2016)[a]. Stable in a cohort from 7 to 70 yr (LC-MS/MS) (Prasad et al., 2014). | Stable | Stable | Stable |
| *SLC22A1*/OCT1 | – | Low expression from neonate up to infant age and increasing to adult age (Prasad et al., 2016)[a]. Increase between 1 and 2 days ($n$ = 7) and 3–4 weeks of age ($n$ = 5) (Western blot) (Hahn et al., 2017). | Lower in fetuses and term newborns than in adults. | Stable | Stable |

[a] 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) (Prasad et al., 2016).

found that the fraction of highly glycosylated OATP1B3 increased with age (Thomson et al., 2016). 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 be 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 (Nies et al., 2009) 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 (Prasad et al., 2016). Moreover, our group previously showed that the *SLC22A1* genotype is related to tramadol disposition in preterm infants, similar to adults (Matic et al., 2016). 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 (Le Vee et al., 2011), 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 h 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 modeling: in vivo hepatic disposition of rosuvastatin was predicted by scaling from individually transfected cell lines by correcting for absolute transporter protein expression levels (Bosgra et al., 2014). 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 (Barrett et al., 2012; Hartmanshenn et al., 2016), or used as appropriate scaling factors to scale between in vitro cell lines and human hepatic expression in adults or pediatric patients.

## 5. 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.

## Authorship contributions

Participated in research design: BDG, ES, MGM, BAEK, RG, CB, JSL, RHNS, WHV, SNW

Organized sample collection: MGM, BAEK, RMV, DT, SNW

Conducted experiments: BDG, ES, HMW, RG, CB, WHV

Contributed new reagents or analytic tools: ES, MMHL, HMW, JSL, WHV, SNW

Performed data analysis: BDG, CB, JSL, JR, SNW

Wrote or contributed to the writing of the manuscript: BDG, ES, MGM, MMHL, BAEK, HMW, RG, CB, JSL, RHNS, JR, RMV, DT, WHV, SNW

## Competing interests

None.

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## Appendix A. Supplementary data

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