Proteomic analysis of the developmental trajectory of human hepatic membrane transporter proteins in the first three months of life

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Hepatic transporter protein expression

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ABC: ATP-binding cassette transporters
BSEP: bile salt efflux pump
CAR: constitutive androstane receptor
CYP3A: Cytochrome P450 3A
GA: gestational age
GLUT1: glucose transporter 1
MCT1: monocarboxylate transporter 1
MDR: multidrug-resistance protein
miRNA: microRNA
MRP: multidrug-resistance like protein
OATP: organic anion transporter polypeptide
OCTN: organic cation/carnitine transporter
PBPK: physiologically-based pharmacokinetic
P-gp: P-glycoprotein
PMA: postmenstrual age
PNA: postnatal age
PXR: pregnane-X-receptor
SLC: solute carrier
SLCO: solute carrier organic anion
UPLC-MS/MS: ultra-performance liquid chromatography tandem mass spectrometry
ABSTRACT

Human hepatic membrane-embedded transporter proteins are involved in trafficking endogenous and exogenous substrates. Even though impact of transporters on pharmacokinetics is recognized, little is known on maturation of transporter protein expression levels, especially during early life. We aimed to study the protein expression of 10 transporters in liver tissue from fetuses, infants and adults. Transporter protein expression levels (ABCB1, ABCG2, ABCC2, ABCC3, BSEP, GLUT1, MCT1, OATP1B1, OATP2B1, and OCTN2) were quantified using UPLC-MS/MS in snap-frozen post mortem fetal, infant and adult liver samples. Protein expression was quantified in isolated crude membrane fractions. The possible association between postnatal and postmenstrual age versus protein expression was studied. We studied 25 liver samples: 10 fetal [median gestational age 23.2 weeks (range 16.4-37.9)], 12 infantile [gestational age at birth 35.1 weeks (27.1-41.0), postnatal age 1 week (0-11.4)], and 3 adult. Exploration of the relationship with age by comparing age groups and correlating age within the fetal/infant age group suggested four specific patterns: stable, low to high, high to low and low-high-low. The impact of growth and development on human membrane transporter protein expression is transporter-dependent. The suggested age-related differences in transporter protein expression may aid our understanding of normal growth and development, and also may impact the disposition of substrate drugs in neonates and young infants.
INTRODUCTION

Membrane-embedded transporter proteins are involved in the transport of a myriad of endogenous and exogenous substances. These transporters can be found in various cell types, such as enterocytes, hepatocytes, brain and kidney cells. By trafficking exogenous substrates, they have an important impact on drug absorption, distribution and excretion of drugs and their metabolites, thereby determining drug efficacy and toxicity. In the hepatocyte, transporters have been identified on both basal and canalicular membranes regulating the uptake and excretion of drugs and their metabolites into and from the systemic circulation as well as excretion into bile (Klaassen and Aleksunes, 2010).

The international transporter consortium has identified a large set of clinically relevant membrane transporters, subdivided in families with similar peptide sequences (International Transporter et al., 2010). Transporters belonging to the ATP-binding cassette (ABC) family are multidrug resistance protein 1 (ABCB1/MDR1/P-gp), multidrug resistance-associated protein 2 and 3 (ABCC2/MRP2, ABCC3/MRP3), breast cancer resistance protein (ABCG2/BRCP), and bile salt export pump (ABCB11/BSEP). To the solute carrier organic anion (SLCO) family belong organic anion transporting polypeptide 1B1 and 2B1 (SLCO1B1/OATP1B1, SLCO2B1/OATP2B1), and are acknowledged to be the most clinically relevant. Other transporter proteins investigated in the current study are glucose transporter 1 (SLC2A1/GLUT1), monocarboxylate transporter 1 (SLC16A1/MCT1), and organic cation/carnitine transporter (SLC22A5/OCTN2), which belong to the solute carrier (SLC) family. This selection of transporters was chosen because they have proven to be important in drug disposition in adults or have shown a large abundancy in human adult liver (Hilgendorf et al., 2007).
Children’s growth and maturation importantly impact the processes involved in the disposition of drugs such as absorption, metabolism and renal excretion (Kearns et al., 2003). It is therefore likely that expression and activity of transporters are also subject to age-related changes, as has been convincingly shown for the drug metabolizing enzymes, as reviewed by de Wildt (de Wildt, 2011). Indeed, animal studies have shown transporter-specific maturation profiles in transporter expression as reviewed by Klaassen, Cui and Brouwer (Klaassen and Aleksunes, 2010; Cui et al., 2012; Brouwer et al., 2015). For example, hepatic mRNA expression of mouse Oatps gradually increases after birth until adult levels at 6 weeks of age (Cheng et al., 2005). In spite of the maturation in animal studies, interspecies differences prevent us to translate animal maturation data to humans (Wang et al., 2015). For humans, only few transporters have been studied and two recent reviews showed that a clear information gap remains on the developmental trajectory of individual transporters in fetuses and young children and the related impact on drug disposition (Brouwer et al., 2015; Mooij et al., 2015). Recently, we showed a transporter dependent maturation profile in mRNA expression of hepatic ABCB1, ABCC2, OATP1B1 and OATP1B3 (Mooij et al., 2014). To predict drug disposition on the basis of these results is a challenge since posttranscriptional variation may result in a discrepancy between mRNA and protein levels, as has been shown for some transporters in human adults (Prasad et al., 2013; Ulvestad et al., 2013). Protein expression data in children are limited. For example, a selection of transporters (ABCC2, ABCG2, ABCB1, OATP1B1, OATP1B3 and OATP2B1) has been quantified in children using quantitative LC-MS methods, but the younger age range (<7 years of age) where most developmental changes occur was not included in these studies (Deo et al., 2012; Prasad et al., 2013; Prasad et al., 2014). Moreover, other pediatric transporter protein data are derived from non-quantitative immunohistochemistry studies (Brouwer et al., 2015).
With the aim to further elucidate developmental changes in human membrane transporters, especially during early life, we conducted an exploratory study using quantitative LC-MS/MS to determine the hepatic protein abundance of a large selection of transporters in liver samples from fetuses, neonates and young infants and relate the findings to adults.
MATERIAL AND METHODS

1. Tissue samples

Post mortem liver tissue samples from autopsy of fetuses and infants were collected by the Erasmus MC Tissue Bank. Tissue was procured at the time of autopsy within 24 hours after death and snap-frozen at -80°C for later research use. The Erasmus MC Research Ethics Board waived the need for ethics approval according to the Dutch Law on Medical Research in Humans. The tissue was collected after parental written informed consent was obtained for both autopsy and the explicit use of the tissue for research, in line with the Dutch guidelines on the secondary use of human tissue. Human adult liver tissue samples (n=3) were a kind gift from Prof. G.M.M. Groothuis, University of Groningen, The Netherlands. They were collected anonymously as surgical waste material from patients after partial hepatectomy because of liver metastasis.

The cohort of selected samples reflected a widespread gestational age (in second and third trimester of pregnancy), as well as postnatal age (up to three months) (age distribution is displayed in Figure 1).

2. Membrane isolation and Liquid Chromatography-Tandem Mass Spectrometry

Isolation of crude membrane fractions from tissue samples was conducted as described by Wisniewski et al., with some minor adaptations (Wisniewski, 2009). In brief, approximately 10 mg of liver tissue 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 100,000g for 30 minutes at 4°C using a LE-80k Centrifuge with SW28 rotor
(Beckman Counter, USA). The pellet, containing crude membrane protein fraction, was resuspended in 500 µL SDT-lysis buffer (0.1 M Tris pH 7.6, 0.1 M DTT, 4% SDS). Approximately 50 µL of cell lysate, corresponding to 1 mg tissue, was incubated at 95°C for 5 minutes and briefly sonicated on ice, mixed with 200 µL of 8 M urea and loaded onto a 30K Amicon Ultra-0.5 centrifugal filter device (Millipore, Darmstadt, Germany) and centrifuged at 14,000g for 15 minutes. After adding another 200 µL 8 M urea the tubes were centrifuged again at 14,000g for 15 minutes. Subsequently, 100 µL of 0.05 M iodoacetamide in 8 M urea in 0.1 M Tris-HCl, pH 8.5 was added and the samples were incubated for 20 minutes at room temperature, and centrifuged at 14,000g for 15 minutes. The resulting concentrate was diluted with 100 µL 8 M urea in 0.1 M Tris-HCl, pH 8.5 and centrifuged (14,000g for 15 minutes) again. This step was repeated twice. Subsequently, 100 µL 0.05 M ammoniumbicarbonate was added to the concentrate and centrifuged (14,000g for 15 minutes). This step was also repeated twice. Proteins were digested into peptides by incubating the samples overnight at 37°C in a 0.05 M ammoniumbicarbonate solution containing 0.5 µg/µL trypsin. The digests were collected into a new collection tube by centrifugation (14,000g for 10 minutes,) and the filter device was rinsed with 50 µL 0.5 M NaCl and centrifuged (14,000g for 10 minutes). The combined filtrate was acidified with 25 µL 0.1% CF₃COOH, desalted on a 7 mm/ 3 mL extraction disk cartridge (3M Empore, Zoeterwoude, The Netherlands) and eluted with 70% acetonitrile. Subsequently, the samples were vacuum dried (SpeedVac) until the solution was vaporized and stored < -70°C until further analysis.

All samples were analyzed using a UPLC coupled to a 6500 QTrap mass spectrometer (AB Sciex). The crude membrane fractions were injected on an Acquity C18 BEH UPLC column and separated using gradient elution. During analysis, the column was maintained at 50°C and the samples were kept at 10 °C. The mass spectroscope was operated in selective reaction mode using electrospray ionization in positive ion mode, with a capillary voltage of 3.3 kV, a source temperature of 150 °C, and a desolvation temperature of 600 °C. Cone voltage and
collision energy were optimized for each compound individually. The multiple reaction monitoring transitions were determined from tandem mass spectra obtained by direct infusion of 0.5 µg/mL. Per peptide three transitions were chosen (Q3-1, Q3-2 and Q3-3) for quantitation and confirmation. The transitions for the different proteins are listed in Table 1. 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 (Table 1). Peak identification and quantification was performed using Analyst software version 1.6.

3. Statistics

Data are presented as median and range, unless indicated otherwise. To describe the variation in protein expression, the relative difference between the lowest and highest observed value was calculated per transporter. Median (range) protein expression per age group (fetuses, infants and adults) was compared using the Kruskal-Wallis test. To further explore the maturation of protein expression, data were visualized in scatterplots with postnatal and postmenstrual age as surrogate markers of maturation. Postnatal age is the time elapsed after birth and postmenstrual age is the time elapsed since the last menstruation (gestational age) plus postnatal age. As data between 3 months of age and adulthood were lacking, modeling the maturation pattern over the whole pediatric age range was not possible. Therefore, the association between transporter protein levels and age was only assessed within the fetal/neonatal group using Spearman’s rank correlation coefficient.

All statistical analyses were performed using IBM SPSS Statistics software (SPSS Statistics for Windows, version 21.0; IBM, Armonk, NY) and a significance level of $p<0.05$ was used. Where relevant, to correct for multiple testing, Bonferroni correction was used ($p<0.05/10$ transporters = $p<0.005$)
RESULTS

Descriptive results

We studied 25 liver samples: 10 of fetuses [median gestational age (GA) 23.2 weeks (range 16.4-37.9)], 12 of infants [postnatal age 1 week (0-11.4), GA at birth 35.1 weeks (27.1-41.0)] and 3 of adults. The clinical diagnoses of the fetuses and infants were: therapeutic abortion for severe genetic disorders (non-metabolic diseases) (n=7), fetomaternal transfusion (n=1), hydrops fetalis (n=1), and intrauterine death for unknown reason (n=1). The clinical diagnoses of the infants were congenital malformations (n=5), viral/bacterial infections (n=4), necrotizing enterocolitis (without liver insufficiency) (n=2), and benign liver tumor (n=1). The adult liver tissue was histologically normal tissue. Information about diagnosis, reason for biopsy or medical history of the three adult patients concerned was not available.

Following the Dutch guideline on secondary use of human tissue, the Tissue Bank only provided the following clinical data: gestational age, postnatal age, gender and main clinical diagnoses. Consequently, data on nutrition or medication use prior to death were unavailable.

Transporter protein expression from fetus to adult

The selected hepatic transporter proteins could be detected in all samples. There was a 10 to 300-fold relative difference in transporter protein abundance between the patients with the lowest and the highest protein expression (Figure 2). The highest variability was seen in GLUT1 protein expression with a nearly 300-fold relative difference. Table 2 shows the median abundance of studied transporters in fetal, infant and adult samples. With exploration of the relationship between age and protein expression four developmental patterns emerged. We describe these patterns, but they should be interpreted with care, as after correction for multiple
testing, only a consistent, significant age-effect remains for BSEP, ABCG2, GLUT1, MCT1. Table 3 shows the four developmental profiles in relation to previous results from other studies on transporter mRNA and protein expression. Protein expression levels in the age groups, postnatal age and postmenstrual age are shown in Table 2, Figure 3 and Figure 4, respectively.

First, in profile I (‘stable’) ABCB1, OATP1B1, OATP2B1 and ABCC2 protein expression levels appeared similar across the age groups and within the fetal and infant groups.

Next, in profile II (‘low to high’) ABCC3, and BSEP protein expression levels appeared low in fetus and higher in infants and adulthood. Median protein levels of ABCC3, and BSEP were approximately 4-fold, and 2-fold lower in fetuses than infants and adults respectively.

The opposite pattern appears for group III (‘high to low’), for ABCG2, GLUT1, and OCTN2, with higher protein abundance early in life than at adult age was observed. This pattern was most striking for GLUT1 with more than 80 fold higher expression in fetus than adult, but less convincing for the ABCG2 and OCTN2 were the difference appears a magnitude 1.5-2 fold. Within the young age group, expression levels appear more strongly correlated with postmenstrual than postnatal age.

Lastly, in profile IV (‘non-linear’) the MCT1 protein expression appeared to display a curvilinear relationship with postnatal age, with a 2-fold higher expression in infants than in both fetuses and adults. Within the fetal and infant groups, the MCT1 protein expression appeared to increase with postmenstrual age but this did not reach significance after correction for multiple testing.
Correlation analysis of transporter protein abundances

To explore potentially shared expression regulation, Spearman correlations of transporter protein expression levels between transporters were performed. In total, the expression levels of 16 transporter-pairs were significantly correlated to each other (Table 4). The strongest correlations were found for the pairs: GLUT1/ABCG2, GLUT1/ABCC2, ABCB1/ABCC2, and ABCG2/OCTN2 ($\rho = 0.70$, $p < 0.0001$; $\rho = -0.65$, $p < 0.001$; $\rho = 0.69$, $p < 0.001$; and $\rho = 0.67$, $p < 0.001$)(Table 4 and Figure 5).
DISCUSSION

This study presents novel data on protein abundance of ten clinically relevant hepatic membrane transporters in the first 3 months of life. From these data, four different developmental profiles of protein abundancy seem to emerge. These results and existing human data from the literature are summarized in Table 3. While we recognize that animal data may provide important insights in maturational changes, we exclusively focus on human data as specific maturation patterns may be very different from animals.

**Stable expression of hepatic transporters: Profile I**

Protein expression of ABCB1, OATP1B1, OATP2B1 and ABCC2 transporters was similar in fetuses, infants and adults. For ABCB1, OATP1B1 and OATP2B1 this adds to current LC-MS/MS data on patients between 7-70 years of age (n=64), in which ABCB1, OATP1B1, and OATP2B1 protein expressions were also stable (Prasad et al., 2014). However, for ABCC2 protein expression an increasing protein expression is previously reported (abstract only) (Tang, 2007).

ABCB1 is the most studied transporter in fetuses and children. Examples of ABCB1 substrate drugs are digoxin, dexamethasone, tacrolimus, and morphine (Hebert, 1997; Ratnapalan et al., 2003; Kimura et al., 2007; Thorn et al., 2009). Interestingly, our finding that ABCB1 protein expression data is consistent in the investigated age-ranges adds to other protein studies using immunoblotting in pediatric postmortem and living donors, in which stable protein expression was found from 0.3-12 years of age (abstract only) (Tang, 2007). In contrast, this apparent stable ABCB1 protein expression differs from the previously reported immature ABCB1 mRNA expression in the first year of life (van Kalken et al., 1992; Miki et al., 2005; Fakhoury et al., 2009; Mooij et al., 2014; Burgess et al., 2015). An explanation between these
discrepant results may reside in the observation in adults that ABCB1 mRNA and protein expressions were not correlated (Ulvestad et al., 2013).

Similar OATP1B1 protein expression in the first months of life and adults contrasted from a study in 78 liver samples from children aged 0-12 years, in which the OATP1B1 protein expression by Western blotting was low from birth until 6 years of age and increased thereafter (abstract only) (Thomson et al., 2013). Yet, a smaller protein study using Western blotting on liver samples from neonates (n=5) and adults (n=5) confirms our finding regarding OATP1B1 (Yanni et al., 2011). OATP1B1 mRNA expression data from several other cohorts do not correspond to our protein data, with low expression in fetuses and infants up to 1 year of age (n=32) (Sharma et al., 2013; Mooij et al., 2014; Burgess et al., 2015).

Stable OATP2B1 protein expression in infants and adults contrasts to lower OATP2B1 mRNA expression in samples of fetuses (n=3) from the second trimester of pregnancy than in samples from adults (n=3) (Sharma et al., 2013).

Our data for ABCC2 are less evident. While fetal expression appeared two-fold lower than adults, this difference was no longer significant after correction for multiple testing. Also within the fetal/infant group, expression was not significantly correlated with either postnatal or postmenstrual age. Using western blotting in children from 0-12 years of age, Tang et al. did show an increase in protein levels (abstract only) (Tang, 2007). In addition, other studies found lower ABCC2 mRNA expression in samples from fetuses and infants up to 1 year of age than in samples from adults (Mooij et al., 2014; Burgess et al., 2015). From 7 years onwards, ABCC2 absolute protein expression levels, also quantified with LC-MS/MS, were stable (Deo et al., 2012). Overall, this suggests an increase in ABCC2 expression in the first years of age, but confirmatory data are needed.
Low to high expression of hepatic transporters: Profile II

The ABCC3, and BSEP transporters showed increasing protein expression from fetus to infant and highest expression in adults.

The BSEP protein expression profile confirms results from the few available mRNA expression studies. One showed approximately 3-fold increasing expression from fetal (n=3) to adult (n=3) liver samples; a second study, found lower expression in neonates than in children older than 7 years of age (Klaassen and Aleksunes, 2010; Sharma et al., 2013). BSEP protein was immunohistochemically detected in second trimester fetuses (Chen et al., 2005).

Lower expression of ABCC3 early in life is not seen in a small study using liver samples of 5 neonates and 5 adults, in which relative ABCC3 protein expression using Western blotting was similar in both groups (Yanni et al., 2011). In contrast, mRNA expression of ABCC3 in 3 fetal liver samples was significantly lower than that in 3 adult liver samples (Sharma et al., 2013) and in perinatal/neonatal liver samples (prenatal to postnatal day 30, n=6; and 0-4 years, n=8) compared to liver samples from children older than 7 years (Klaassen and Aleksunes, 2010).

High to low expression of hepatic transporters during childhood: Profile III

The ABCG2, GLUT1, and OCTN2 transporters appear to be higher early in life and lower at adult age. This adds to current data of stable protein expression from 7 years onwards (Prasad et al., 2013).

The approximately two-fold higher ABCG2 protein expression early in life does not correspond to the stable ABCG2 protein expression found using Western blotting in 5 neonatal livers and 5 adult livers (Yanni et al., 2011). ABCG2 protein visualized by immunohistochemistry staining showed weak coloring in first trimester fetal hepatocytes, while adult liver showed
strong ABCG2 staining (Konieczna et al., 2011). On the other hand, stable ABCG2 mRNA expression with age was found in two studies; in fetal, pediatric, and adult (n=90) liver samples and a study comparing 3 fetal and 3 adult livers (Sharma et al., 2013; Burgess et al., 2015).

To the best of our knowledge, there are no human developmental expression data available for GLUT1 and OCTN2. The same holds for MCT1, which forms the fourth developmental profile group with non-linear expression profile amongst the three age categories.

The statistical analysis of these data to explore the relation with age posed an important challenge. While these patterns point towards age-dependent changes in transporter expression, our data sample was too small to explore the exact developmental patterns using different statistical models with an acceptable degree of certainty. Historically, pediatric dosing has been based on the assumption that adult doses could be adjusted linearly based on body weight. We now know that for most drugs this approach is inadequate as it does not take into account immaturity of drug disposition processes including metabolism and renal function. The maturation of these processes does not follow a linear pattern and is very specific for individual processes, even for individual metabolizing enzymes. The complexity of these individual patterns has been shown by the group of Knibbe et al., which validated for population PK models a body-weight derived function in which the allometric exponent of the clearance model decreased in a sigmoidal manner with bodyweight (Wang et al., 2013). For our and future transporter data in pediatrics or even beyond, a similar approach may be considered to optimally describe the developmental patterns.
Variability in transporter expression

This exploratory study confirms earlier reports of transporter-specific discrepancy between mRNA and protein expression. Our ABCB1, ABCG2, OATP1B1, OATP2B1 protein developmental patterns do not seem to correspond to earlier mRNA expression data (Yanni et al., 2011; Sharma et al., 2013; Mooij et al., 2014). This discrepancy shows the relative weakness of relying solely on mRNA expression data to understand transporter maturation. An explanation could be posttranscriptional changes – for example mediated by microRNAs – on account of which mRNA does not correspond to protein expression (Koturbash et al., 2012). Recently, developmental changes in hepatic miRNA expression have been shown, and for some, miRNA expression was correlated to mRNA expression of drug disposition genes (Burgess et al., 2015). The interpretation of protein data should also be done with care, as transporter protein expression itself may not necessarily translate to transporter activity. Glycosylation, phosphorylation, and ubiquination, might alter proteins into active or non-active forms (Klaassen and Aleksunes, 2010).

It can be speculated that the maturation of transporter expression is in line with the physiological transporter function with respect to growth and development. For example, transporters that are involved in energy supply in fetal life may be up regulated in fetuses and early in life. Glucose transporter GLUT1 and L-carnitine (molecule that shuttles long-chain fatty acids to the mitochondria for oxidation) transporter OCTN2, are probably both essential for fetal energy supply, which seems to tally with our findings of high protein expression in fetuses and early in life. On the other hand, transporters involved in the uptake and efflux of steroids and bilirubin (i.e. OATPs, MRP2), may show less variability throughout life.

We hypothesized that pairwise-correlation in transporter expression might reveal a common developmental origin and point towards interlinked physiological processes. We found
strong correlations for the following pairs: GLUT1/ABCG2, GLUT1/ABCC2, ABCB1/ABCC2, and ABCG2/OCTN2. Similarly, in adult liver samples correlations between multiple CYP and UGT protein expression were demonstrated (Achour et al., 2011; Achour et al., 2014a; Achour et al., 2014b). To our knowledge, these inter-transporter correlations at the protein level have not been described previously. Many drugs have been shown to be substrates for multiple transporters, which may result in compensatory pathways in case a specific transporter is inactive due to genetic variation or drug-drug interactions. In addition, hepatocyte-hopping of substrates using different transporters may enhance efficient hepatic drug clearance (Iusuf et al., 2012). Therefore, when using transporter expression data to predict drug disposition it is recommended to consider drug transport, and also drug metabolism as an interrelated biological system.

Variability in transporter expression may also be explained by other factors than age, such as disease, genetic heterogeneity, gender, drug-drug and drug-food interactions. This may explain some of the discrepancy between studies, as the sample origin and clinical characteristics are often only sparsely described. In human hepatocytes, inflammation via interferon-γ resulted in reduced mRNA expression of ABCB1, ABCC2, OATP1B1 and OATP2B1 (Le Vee et al., 2011). More specifically in pediatric patients (1-2 months of age) with biliary atresia, hepatic ABCC2, BSEP, ABCB4, OATP1B1, OATP1B3, but not ABCC3 mRNA expression differed significantly from that in both fetuses and adults (Chen et al., 2008). Genetic polymorphisms are well known to govern drug efficacy and toxicity; variants in OATP1B1 and ABCG2 encoding genes, for example, were found to influence statin efficacy/toxicity (DeGorter et al., 2012). Gender did not impact protein expressions of ABCG2, ABCB1, ABCC2, OATP1B1, OATP1B3 and OATP2B1 (Deo et al., 2012; Prasad et al., 2013; Prasad et al., 2014). Due to the limited sample size and the lack of detailed clinical data, we could not incorporate other covariates than age in our study.
While this explorative study adds importantly to existing pediatric transporter data by studying protein levels in the age group where most developmental changes are expected for 10 transporters, some limitations are present. The sample size was small and most samples are from young infants up to 3 months, so that possible effects of maturation later in pediatric life could not be studied. On the other hand, most developmental changes are expected in the first year of life. Second, all liver samples were collected postmortem, within 24h, but the exact time was unknown. While the stability of hepatic proteins following death has been previously studied and shown stable enough to allow meaningful studies (Finger et al., 1987), the time gap might have introduced further variability in the results. Further studies should be aimed to extend the pediatric age range, extend the selection of transporters, preferably involve larger sample size, and combining transporter expression with regulator proteins and genotyping.

Eventually, transporter maturation profiles and their interrelation may serve as input for physiologically-based pharmacokinetic (PBPK) models, which can be applied to use existing juvenile infant and human adult pharmacokinetic information to predict pharmacokinetics and drug response in children (Johnson et al., 2006; Bosgra et al., 2014).

In conclusion, we revealed age-dependent differences in absolute protein expression levels of ten different hepatic transporter proteins, with four developmental patterns emerging. These findings are important as they strongly suggest that disposition of drugs and endogenous transporter substrates will be subject to age-related changes, which could impact the efficacy and safety of drugs in the first months of life.
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AUTHORSHIP CONTRIBUTIONS

- Participated in research design: Mooij, van de Steeg, de Koning, Vaes, de Wildt
- Conducted experiments: van de Steeg, Wortelboer, Vaes
- Contributed new reagents or analytic tools: van de Steeg, Wortelboer, Vaes, de Wildt
- Performed data analysis: Mooij, van Rosmalen, van Groen, de Wildt
- Wrote or contributed to the writing of the manuscript: Mooij, van de Steeg, van Rosmalen, Windster, de Koning, van Groen, Tibboel, Wortelboer, de Wildt
REFERENCES


monkey, and rat as determined by quantitative proteomics. *Drug Metab Dispos* **43**:367-374.


FIGURE LEGENDS

FIGURE 1: Age distribution in fetal and infantile samples.

FIGURE 2: Variability in protein expression.

Legend: Each dot represents an individual liver sample, the bars present median and range


Legend: Triangles represent individual fetal samples, circles represent pediatric samples, and squares represent adult liver samples. Corresponding Spearman’s rank correlations ($r^2$) between fetal/infant age and protein levels are shown in each graph. Organized by developmental profile groups (I to IV).

FIGURE 4: Transporter-specific postmenstrual maturation of protein expression.

Legend: Closed circles represent individual fetal samples and open circles represent pediatric liver samples. Corresponding Spearman’s rank correlations ($r^2$) are shown in each graph. Organized by developmental profile groups (I to IV).

FIGURE 5: Examples of inter-transporter relationship of protein expression.

Each circle represents an individual liver sample. These four pairs represent the strongest inter-transporter correlation. Corresponding Spearman’s rank correlation ($r$) are shown in each graph.
TABLE 1 Multiple reaction monitoring (MRM) transitions of the used peptides and the corresponding internal standards (AQUA)

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<th>Name</th>
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<th>Peptide sequence\textsuperscript{a}</th>
<th>Molecular weight</th>
<th>Q1</th>
<th>Q3-1</th>
<th>Q3-2</th>
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* AQUA: Amino acid presented in **bold** is labelled with $^{13}$C and $^{15}$N.
### TABLE 2 Hepatic protein expressions

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<thead>
<tr>
<th>Age group vs expression</th>
<th>Profile I</th>
<th>Profile II</th>
<th>Profile III</th>
<th>Profile IV</th>
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<tr>
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<td>‘Stable’</td>
<td>‘Low to high’</td>
<td>‘High to low’</td>
<td>‘Non-linear’</td>
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<tr>
<td>Fetuses N = 10</td>
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<tr>
<td>Infants N = 12</td>
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</tr>
<tr>
<td>Adults N = 3</td>
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</table>

<table>
<thead>
<tr>
<th>Protein</th>
<th>Fetuses (range)</th>
<th>Infants (range)</th>
<th>Adults (range)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>OATP1B1</td>
<td>17.6 (5.4-35.6)</td>
<td>22.8 (9.5-47.9)</td>
<td>34.6 (21.5-57.5)</td>
<td>0.126</td>
</tr>
<tr>
<td>OATP2B1</td>
<td>42.2 (29.3-59.3)</td>
<td>38.5 (27.2-46.3)</td>
<td>44.9 (30.1-48.9)</td>
<td>0.580</td>
</tr>
<tr>
<td>ABCB1</td>
<td>79.2 (23.2-117.7)</td>
<td>107.9 (60.6-150.6)</td>
<td>96.9 (93.5-103.4)</td>
<td>0.065</td>
</tr>
<tr>
<td>ABCC2</td>
<td>8.8 (5.5-9.9)</td>
<td>12.6 (5.3-43.9)</td>
<td>19.8 (15.5-22.2)</td>
<td>0.010</td>
</tr>
<tr>
<td>ABCC3</td>
<td>5.4 (1.6-9.9)</td>
<td>21.9 (3.6-32.8)</td>
<td>23.1 (15.9-23.7)</td>
<td>0.012</td>
</tr>
<tr>
<td>BSEP</td>
<td>18.4 (11.4-33.7)</td>
<td>35.9 (10.6-49.8)</td>
<td>47.7 (40.3-67.0)</td>
<td>0.005*</td>
</tr>
<tr>
<td>ABCG2</td>
<td>4.0 (2.2-7.0)</td>
<td>2.7 (1.5-4.2)</td>
<td>1.7 (0.8-2.2)</td>
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<tr>
<td>GLUT1</td>
<td>434.2 (49.1-1413.3)</td>
<td>108.7 (39.0-255.4)</td>
<td>5.2 (5.1-7.7)</td>
<td>&lt;0.000*</td>
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<tr>
<td>OCTN2</td>
<td>6.3 (4.1-8.9)</td>
<td>4.4 (1.9-6.6)</td>
<td>3.0 (2.2-4.8)</td>
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<tr>
<td>MCT1</td>
<td>12.9 (10.0-20.0)</td>
<td>20.3 (10.8-39.0)</td>
<td>9.8 (8.9-12.4)</td>
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Data are expressed as median (range) protein expressions in pmol/g tissue, p-value. * significant after Bonferroni correction (0.05/10 = 0.005).
TABLE 3 Summary table of results in relation to literature data

<table>
<thead>
<tr>
<th>Family</th>
<th>Transporter</th>
<th>Protein expression profile group</th>
<th>Literature data: Age vs mRNA expression</th>
<th>Literature data: Age vs protein expression</th>
<th>Current data: postnatal age vs protein expression</th>
<th>Current data: postmenstrual age vs protein expression</th>
<th>Current data: Inter-transporter correlation of protein expression</th>
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</thead>
<tbody>
<tr>
<td>Solute carrier organic anion (SLCO) family</td>
<td>OATP1B1</td>
<td>Profile I ‘Stable’</td>
<td>Low-high: with low expression in fetuses and infants up to 1 year of age (n=32) (Mooij et al., 2014), and low expression in fetal (n=30) versus pediatric and adult samples (n=30 each)(Burgess et al., 2015), and again lower mRNA expression in 3 fetuses than 3 adults (Sharma et al., 2013).</td>
<td>Stable: In neonates and adults (n=10, western blotting) expression was similar (Yanni et al., 2011). In 7-70 years of age, OATP1B1 (n=64, LC-MS/MS) protein expression was stable (Prasad et al., 2014).</td>
<td>Similar: PNA &lt;3 months vs adults</td>
<td>Stable from PMA 16.43 to 51.29 weeks</td>
<td>ABCB1*, ABCC2*, BSEP**, ABCG2**, GLUT1*</td>
</tr>
<tr>
<td>Gene</td>
<td>Expression Patterns</td>
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<tr>
<td>OATP2B1</td>
<td>Low-high: OATP2B1 mRNA expression was lower in 3 fetuses from second trimester of pregnancy than in 3 adults (Sharma et al., 2013). Stable: In 7-70 years of age, OATP2B1 (n=64, LC-MS/MS) protein expressions was stable (Prasad et al., 2014). Similar: PNA &lt;3 months vs adults Stable from PMA 16.43 to 51.29 weeks</td>
<td></td>
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</tr>
<tr>
<td>ABCB1</td>
<td>Low-high: Increase during fetal life, continues postnatal to reach adult levels around 1 year of age (van Kalken et al., 1992; Miki et al., 2005; Fakhoury et al., 2009; Mooij et al., 2014; Burgess et al., 2015). Stable: Stable protein expression from 0.3-12 years of age (n=65, western blotting, abstract only) (Tang, 2007) Similar: PNA &lt;3 months vs adults Stable from PMA 16.43 to 51.29 weeks MCT1*</td>
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<tr>
<td>ABCC2</td>
<td>Low-high: 4-fold higher in pediatric and adult than fetal liver samples (Burgess et al., 2015). Low ABCC2 mRNA expression up to 1 year of age where it reaches adult levels (Mooij et al., 2014). Low-high: Increasing protein expression in 0.3-12 years of age (n=65, western blotting (Tang, 2007). From 7 years of age onwards stable expression (n=51, LC-MS/MS) (Deo et al., 2012). Similar: PNA &lt;3 months vs adults Stable from PMA 16.43 to 51.29 weeks ABCB1**, GLUT1**</td>
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<tr>
<td>ABCC3</td>
<td>Low-high</td>
<td>ABCC3 mRNA expression was significantly lower in livers of 3 fetuses than 3 adults and in perinatal/neonatal (prenatal to postnatal day 30, n=6; and 0-4 years, n=8) compared to older than 7 years liver samples (Klaassen and Aleksunes, 2010).</td>
<td>Low-high: ABCC3 mRNA expression was significantly lower in livers of 3 fetuses than 3 adults (Sharma et al., 2013) and in perinatal/neonatal (prenatal to postnatal day 30, n=6; and 0-4 years, n=8) compared to older than 7 years liver samples (Klaassen and Aleksunes, 2010).</td>
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<td>Low-high</td>
<td>ABCB1*, ABCC2**, GLUT1**</td>
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<tr>
<td>BSEP</td>
<td>Low-high</td>
<td>BSEP mRNA expression increased from fetal (n=3) to approximately 3-fold in adult (n=3) livers, in another study, expression was lower in neonates than in children older than 7 years (Klaassen and Aleksunes, 2010; Sharma et al., 2013).</td>
<td>BSEP mRNA expression increased from fetal (n=3) to approximately 3-fold in adult (n=3) livers, in another study, expression was lower in neonates than in children older than 7 years (Klaassen and Aleksunes, 2010; Sharma et al., 2013).</td>
<td>Low-high</td>
<td>Low-high</td>
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<td>Stable: ABCG2 mRNA expression was found; in fetal (n=30, second trimester of</td>
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<td>High-low</td>
<td>OATP1B1**, OCTN2**</td>
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Solute carrier (SLC) family

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<td>MCT1</td>
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<td>Non-linear</td>
<td>Low-high</td>
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</table>

* P<0.05, ** P<0.01

pregnancy), pediatric (n=30, age 1-17 years) and adult (age 28-80 years) liver samples and in a study comparing 3 fetal and 3 adult livers (Sharma et al., 2013; Burgess et al., 2015). (n=10, western blotting) (Yanni et al., 2011). And in 7-70 years of age, ABCG2 (n=56, LC-MS/MS), protein expressions was stable (Prasad et al., 2013 ). ABCG2 protein immunohistochemistry staining showed weak coloring in first trimester fetal hepatocytes, while adult liver showed strong ABCG staining (Konieczna et al., 2011).
TABLE 4 Correlations of transporter protein expressions

<table>
<thead>
<tr>
<th>Profile</th>
<th>OATP1B1</th>
<th>OATP2B1</th>
<th>ABCB1</th>
<th>ABCC2</th>
<th>ABCC3</th>
<th>BSEP</th>
<th>ABCG2</th>
<th>GLUT1</th>
<th>OCTN2</th>
<th>MCT1</th>
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<tbody>
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<td>OATP1B1</td>
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<td>-0.65***</td>
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<td>0.70***</td>
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<td>IV 'Non-linear'</td>
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</tbody>
</table>

*P<0.05, **P<0.01, ***P<0.001
Age distribution

Figure 1

Gestational age in weeks

Postnatal age in weeks
Transporter protein expression

Figure 2

proteins expressed in pmol/g tissue: ABCG2, OCTN2, ABCC3, ABCC2, MCT1, BSEP, OATP1B1, OATP2B1, ABCB1, GLUT1
Figure 3

Profile I 'Stable'

Profile II 'Low to high'

Profile III 'High to low'

Profile IV 'Non-Linear'

DMD Fast Forward. Published on April 21, 2016 as DOI: 10.1124/dmd.115.068577 at ASPET Journals on October 20, 2017 dmd.aspetjournals.org Downloaded from
Profile I 'Stable'

Profile II 'Low to high'

Profile III 'High to low'

Profile IV 'Non-Linear'

Figure 4
Figure 5

A) $r = 0.69$, $P < 0.0001$

B) $r = -0.65$, $P < 0.0001$

C) $r = 0.70$, $P < 0.0001$

D) $r = 0.67$, $P < 0.0001$