The AAPS Journal (2019) 21: 88
DOI: 10.1208/s12248-019-0359-1

Research Article

Optimized Renal Transporter Quantification by Using Aquaporin 1 and Aquaporin 2 as Anatomical Markers: Application in Characterizing the Ontogeny of Renal Transporters and Its Correlation with Hepatic Transporters in Paired Human Samples

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Received 29 April 2019; accepted 27 June 2019; published online 11 July 2019

Abstract. Renal transporters, which are primarily located in the proximal tubules, play an important role in secretion and nephrotoxicity of drugs. The goal of this study was to characterize the age-dependent protein abundance of human renal transporters. A total of 43 human kidneys, 26 of which were paired with livers from the same donors, were obtained and classified into three age groups: children (< 12 years), adolescents (12 to < 18 years), and adults (> 18 years). Protein abundance of kidney-specific anatomical markers, aquaporins 1 and 2 (markers of proximal and distal/collecting tubules, respectively), and 17 transporters was quantified by LC-MS/MS proteomics. Six out of 43 kidney samples were identified as outliers (Grubbs' test) that were significantly different from the others with relatively higher aquaporin 2 to aquaporin 1 ratio, indicating that these cortex samples were likely contaminated by medulla (representing distal/collecting tubules). No significant age-related changes (age > 1 year) were observed for renal transporter abundance, albeit OCT2 abundance was modestly higher (< 50%) in adolescents than that in adults. Higher protein-protein correlation between transporters was observed in the kidney but abundance of transporters between tissues was not correlated. The use of aquaporins 1 and 2 provides a method for identifying kidney cortex with significant contamination from medulla containing distal and collecting tubules. The abundance and protein-protein correlation data can be used in physiologically based pharmacokinetic (PBPK) modeling and simulation of renal drug disposition and clearance in pediatric populations.

KEY WORDS: ontogeny; renal transporters; liver; kidney; quantitative proteomics; paired samples.

INTRODUCTION

The kidney, along with the liver, plays an important role in eliminating therapeutic drugs and their metabolites. Over 30% of prescribed drugs are primarily cleared by the kidney unchanged (1). The renal elimination process consists of glomerular filtration and transporter-mediated active tubular secretion and reabsorption. These transporters are mainly located in the basolateral and luminal membranes of proximal tubules, which are enriched in the cortex (2) (Fig. 1). The clinical importance of renal transporters in drug secretion, reabsorption, and toxicity has been shown for several prescribed drugs (1–4). For example, penicillin derivatives and other anionic drugs are cleared by anionic transporters in the kidney, which can be inhibited by probenecid leading to higher plasma concentration of these drugs (4). The anticancer drug cisplatin, which is widely used in adults and children, is transported into kidney epithelial cells by organic cation transporter 2 (OCT2). Patients with genetic polymorphism of OCT2 (rs316019) are protected from cisplatin-induced nephrotoxicity (5,6). On the luminal side, the efflux transporter multidrug resistance protein 1 (MDR1) is involved in the urinary excretion of the immunosuppressant drug, tacrolimus. MDR1 polymorphisms in pediatric patients have been associated with nephrotoxicity, which is postulated to be due to the accumulation of tacrolimus in the kidney (7). Thus, it is important to understand transporter abundance in the human kidneys to predict the transporter-mediated drug secretion or reabsorption, drug-drug interaction, and nephrotoxicity.

Electronic supplementary material The online version of this article (https://doi.org/10.1208/s12248-019-0359-1) contains supplementary material, which is available to authorized users.

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Using liquid chromatography-tandem mass spectrometry (LC-MS/MS) proteomics, our laboratory previously quantified the protein abundance of renal transporters in the adult human kidneys (8), which was applied for *in vitro* to *in vivo* extrapolation (IVIVE) of transporter-mediated clearance of metformin (9). Currently, there is little information on renal transporter abundance, at the mRNA, protein, or activity level in the human developing the kidney (10). This is restricted by the limited accessibility to human pediatric kidney samples and by the poor quality of antibodies for protein quantification. Therefore, the major goal of this study was to characterize the ontogeny of renal transporters using our acquired kidney samples by quantitative proteomics and to compare correlation of transporter abundances in the kidney relative to the liver for which ontogeny data are available (11).

Anatomically, the kidney consists of cortex and medulla, and renal transporters are primarily located in the proximal tubules that are enriched in the cortex. Given that the relative composition of cortex *versus* medulla in frozen tissue could vary depending on sample collection and dissection, interpretation of ontogeny data could be confounded by contamination of cortical samples with medulla, leading to underestimation of transporter abundance. Therefore, the first aim of this study was to develop an LC-MS/MS-based method to detect medullary contamination in the archived human kidney samples using anatomical markers, aquaporin 1 (AQP1) and aquaporin 2 (AQP2), which are localized primarily in the proximal tubules and distal/collecting tubules of the kidney, respectively (12,13). The method was then applied to quantify the ontogeny of 17 renal transporters.

Although developmental trajectories of transporter expression in the kidney are not well characterized, age-dependent changes in the abundance of hepatic OCT1, OATP1B3, MDR1, and MRP3 have been reported in pediatric and the adult livers (11). To determine whether renal transporter expression correlates with the liver transporter ontogeny, we investigated transporter abundance in paired the kidney and liver samples. The quantified protein abundances of renal and hepatic transporters could be applied to develop physiologically based pharmacokinetics (PBPK)-based prediction models across various age groups.

MATERIALS AND METHODS

Chemicals and Reagents

Bovine serum albumin (BSA), iodoacetamide (IAA), dithiothreitol (DTT), membrane protein extraction kit, and trypsin were purchased from Thermo Fisher Scientific (Rockford, IL). The synthetic light peptide and isotope-labeled heavy peptides were purchased from New England Peptides (Boston, MA) and Thermo Fisher Scientific (Rockford, IL), respectively. Ammonium bicarbonate buffer (98% purity) was procured from Acros Organics (Geel, Belgium). Chloroform, methanol, formic acid, and MS-grade acetonitrile were purchased from Fisher Scientific (Rockford, IL). Human serum albumin was purchased from Calbiochem (Billerica, MA). All other chemicals and reagents, unless indicated otherwise, were purchased from Sigma-Aldrich (St. Louis, MO).

Procurement of Human Kidney and Liver Tissues

Human kidney and liver tissues were obtained from the Eunice Kennedy Shriver National Institute of Child Health and Human Development Brain and Tissue Bank for Developmental Disorders at the University of Maryland, including 43 kidney samples (17 African American, 26 Caucasian), of which 26 were paired with livers collected from the same donors. The detailed demographic information is provided in Supplementary Table S1. These kidney and liver samples were grouped into four age categories based on FDA age classification: infants (< 1 year), children (1 to < 12 years), adolescents (12 to < 18 years), and adults (> 18 years) (Table I). The use of these tissues was declared non-human subjects research by the Institutional Review Boards of Children's Mercy Hospital (Kansas, MO) and approved by the University of Washington IRB (Seattle, WA).

Membrane Protein Extraction and Trypsin Digestion

The kidney and liver tissues (~ 60 mg) were homogenized, and the membrane fractions were isolated using Mem-PER™ Plus Membrane Protein Extraction Kit (Thermo Fisher, Rockford, IL) as described previously (8). The total

Table I. Demographic Information of Human Kidney and Paired Liver Samples

Number of samples	43 (43 kidney and 26 paired liver samples)	
Age		
Mean	15 years 22 days	
Min	206 days	
Max	35 years 364 days	
Sex		
Male	27	
Female	16	
Age category	Kidney	Liver
Infants (< 1 year)	1 (male)	1 (male)
Children (1 year to < 12 years)	12 (6 males, 4 females)	4 (3 males, 1 females)
Adolescents (12 years to < 18 years)	13 (8 males, 5 females)	5 (4 males, 1 females)
Adults (> 18 years)	17 (10 males, 7 females)	16 (10 males, 6 females)

membrane protein concentration was determined by bicinchoninic acid (BCA) assay (Pierce Biotechnology, Rockford, IL) and was then diluted to a working concentration of 2 mg protein per ml for quantitative proteomics.

Quantitative Proteomics Sample Preparation and LC-MS/MS Analysis

Eighty microliters of human tissue membrane proteins (2 mg/ml) was incubated with 10 μl of dithiothreitol (250 mM), 30 μl of ammonium bicarbonate buffer (100 mM, pH 7.8), 20 μl of BSA (0.02 mg/ml), and 10 μl of human serum albumin (10 mg/ml) at 95°C for 10 min. After cooling down to room temperature, 20 μl of iodoacetamide (500 mM) was added to the mixture and incubated at room temperature for 30 min in the dark. To concentrate the sample, ice-cold methanol (0.5 ml), chloroform (0.1 ml), and water (0.4 ml) were added to each sample. After centrifugation at 16,000×*g* for 5 min at 4°C, the pellet was washed once with ice-cold methanol (0.5 ml) and centrifuged at 8000×*g* for 5 min at 4°C. The pellet was resuspended with 60 μl of ammonium bicarbonate buffer (50 mM). Finally, the protein sample was digested with 20 μl of trypsin at 1:10 trypsin:protein ratio (*w*/*w*) and incubated for 16 h at 37°C with mixing at 300 rpm. The digestion reaction was quenched by 20 μl of chilled heavy internal standard (dissolved in 80% acetonitrile with 0.5% formic acid) and centrifuged at 4000×*g* for 5 min at 4°C. For each sample, 5 μl of the supernatant was introduced into the LC-MS/MS system for analysis. All samples were digested and processed in triplicate.

The surrogate peptides of transporters (Supplementary Table S2) were quantified in the digested samples using a validated LC-MS/MS method (6,9). Renal transporters quantified were OAT1, OAT2, OAT3, OAT4, OCT2, OCT3, OATP4C1, OCTN1, OCTN2, MDR1, MRP2, MRP3, MRP4, URATE, BCRP, MATE1, and MATE2K. Quantitative proteomics was also applied to the paired livers to quantify protein abundance of OATP1B1, OATP1B3, OATP2B1, OATP1A2, OAT2, OCT1, OCT3, BSEP, MDR1, BCRP, MRP2, MRP3, MRP4, and MATE1. Light peptides represent analytes and the corresponding heavy peptides were used as internal standards. Pooled total membrane fractions isolated from liver and kidney tissues were used as a calibrator for estimation of abundances of individual transporters. The calibration curve range and linearity was verified by serial dilutions of the studied transporter peptide standards. The abundances of transporters were measured by LC-MS/MS (SCIEX Triple Quadrupole 6500 system (Framingham, WA) coupled to an ACQUITY UPLC system (Waters Technologies, Milford, MA)). Five microliters of each sample was injected to the column (ACQUITY UPLC HSS T3 1.8 μm, C_{18} 100A; 100 × 2.1 mm, Waters, Milford, MA). The column and method were used with a gradient mobile phase (0.3 ml/min) consisted of 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B). The LC-MS/MS data were analyzed by Skyline software.

Data Analysis

Drug transporters in the kidney are enriched in the cortex but not in the medulla. To confirm if our archived kidney tissue samples were contaminated by medulla, we used ratio of aquaporin 2 (AQP2) to AQP1 to identify the outliers using Grubbs' test ($p < 0.01$). The outliers were excluded from further analysis. The kidney proteomics data are presented as absolute or relative data. The absolute transporter protein abundances were presented at means ± S.D., in the unit of picomole per gram of tissue, which was derived from the transporter protein abundance (picomole per milligram of protein) multiplied by milligram of total membrane protein per gram of tissue (TM-PPGT) (Supplementary Fig. S1). Relative kidney protein abundance data were generated by normalizing abundance data by AQP1 data. Liver transporter abundance data were expressed as pmol per gram of liver tissue. Protein abundance at different age groups were compared by the Kruskal-Wallis test, followed by Dunn's multiple comparison test. Protein-protein expression correlation was estimated using the Spearman correlation, with p value < 0.05 considered as significant.

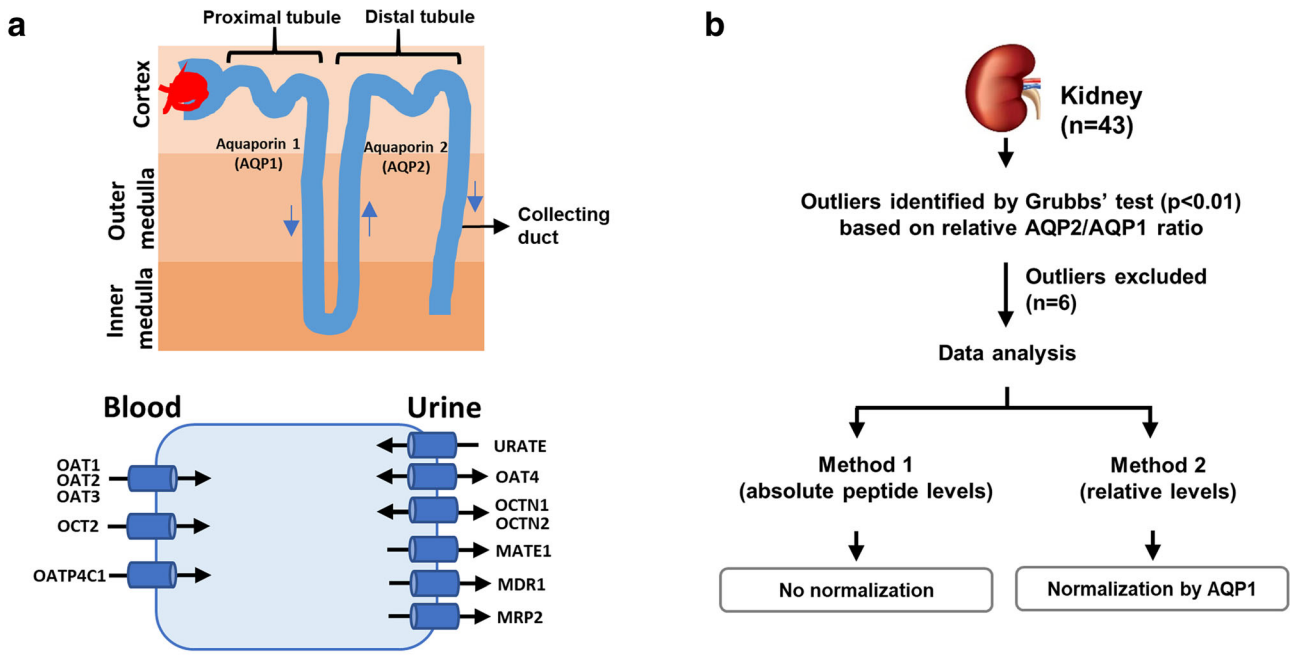
RESULTS

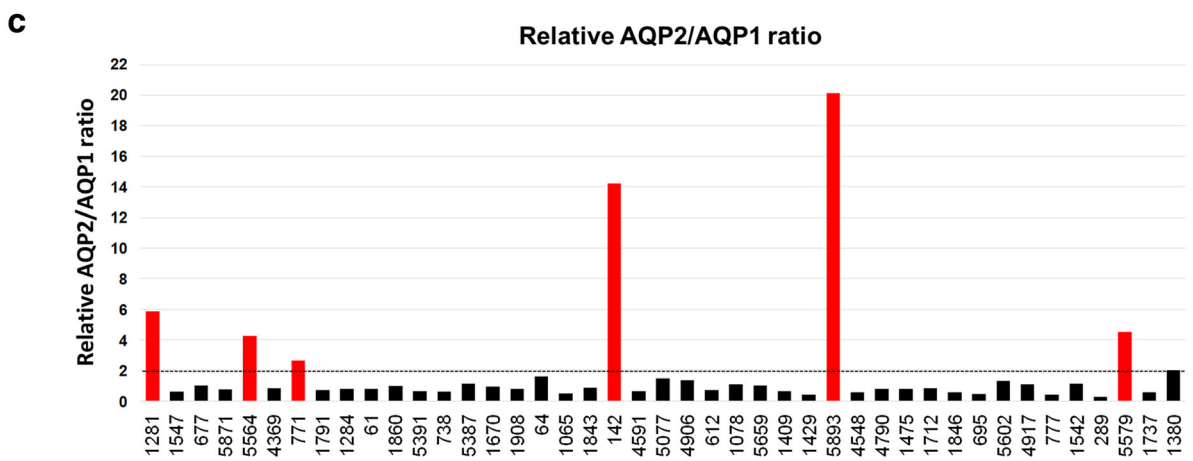
Aquaporin 2 to Aquaporin 1 Ratio as a Marker of Medulla Contamination in Cortex

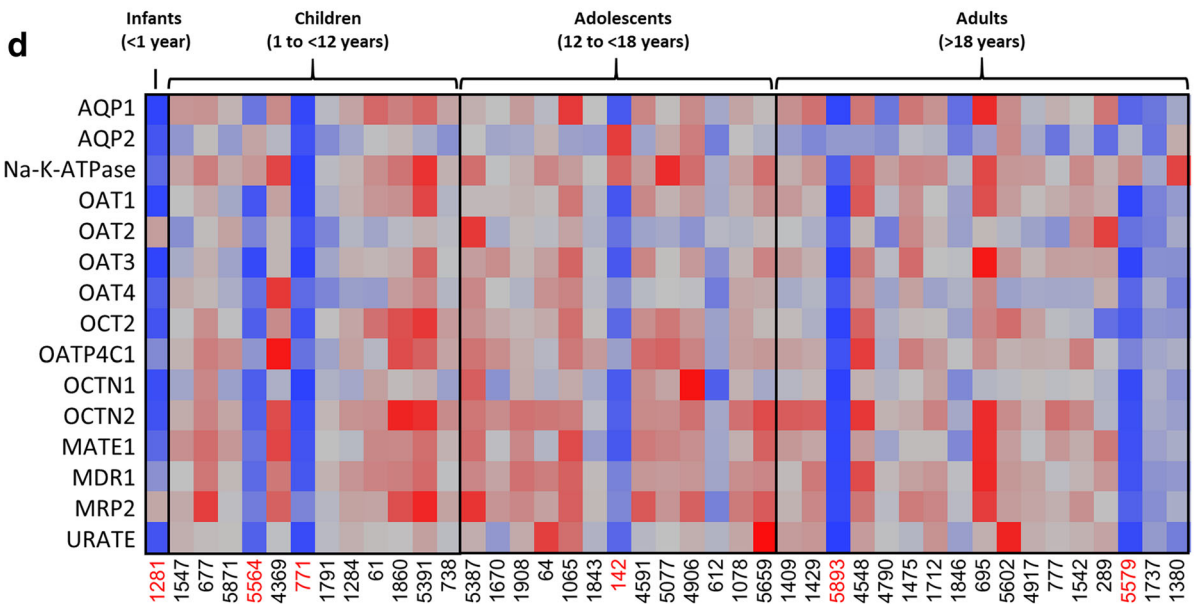
Because renal transporters are primarily located at the proximal tubules, the protein abundances of AQP1 (a marker of proximal tubules) and AQP2 (a marker of distal/collecting tubules) were quantified in the present study to ensure that all "kidney" samples were cortex, as requested, and lacked significant contamination with medulla (Fig. 1a, b). Six out of 43 kidney samples were identified as outliers, which were significantly different from the others with relatively higher AQP2/AQP1 ratio (Grubbs' test, $p < 0.01$) (Fig. 1c). The protein abundances of renal transporters were consistently lower in these six samples, which confirmed that these cortex samples were likely contaminated by medulla (Fig. 1d).

Age-Dependent Protein Abundance of Renal and Hepatic Transporters

In the kidney cortex, the protein abundance of OCT2 was found to be the highest in the kidney cortex, followed by MATE1, OAT1, OAT3, MDR1, MRP2, OAT2, OCTN1, OCTN2, OATP4C1, and OAT4 (Fig. 2a, b). No significant age-related changes were observed for these transporters, albeit OCT2 abundance was modestly higher in adolescents than in adults (< 1.5-fold). There was no significant association of sex with renal transporter abundance (Supplementary Fig. S2). Although OCT3, BCRP, MRP3, MRP4, and MATE2K were detected in the kidney samples, they were below the lower limit of quantification (LLOQ) in majority of samples (data not shown). In the liver, consistent with our previous data, the protein abundance of MRP3 was significantly lower (~ 2-fold) in children *versus* adults. There was no significant age-related changes observed for other hepatic transporters in the liver from children at > 1 year old (Supplementary Fig. S3). Due to the limited number of female liver samples, sex-specific effects on the hepatic transporter abundance could not be tested in


a
Proximal tubule
Distal tubule
Cortex
Outer medulla
Inner medulla
Aquaporin 1 (AQP1)
Aquaporin 2 (AQP2)
Collecting duct
Blood
Urine
OAT1
OAT2
OAT3
OCT2
OATP4C1
URATE
OAT4
OCTN1
OCTN2
MATE1
MDR1
MRP2
b
Kidney (n=43)
Outliers identified by Grubbs' test (p<0.01) based on relative AQP2/AQP1 ratio
Outliers excluded (n=6)
Data analysis
Method 1 (absolute peptide levels)
Method 2 (relative levels)
No normalization
Normalization by AQP1


c
Relative AQP2/AQP1 ratio
Relative AQP2/AQP1 ratio
22
20
18
16
14
12
10
8
6
4
2
0
1281 1547 677 5871 5564 4369 771 1791 1284 61 1860 5391 738 5387 1670 1908 64 1065 1843 142 4591 5077 4906 612 1078 5659 1409 1429 5893 4548 4790 1475 1712 1846 695 5602 4917 777 1542 289 5579 1737 1380


d
Infants (<1 year)
Children (1 to <12 years)
Adolescents (12 to <18 years)
Adults (>18 years)
AQP1
AQP2
Na-K-ATPase
OAT1
OAT2
OAT3
OAT4
OCT2
OATP4C1
OCTN1
OCTN2
MATE1
MDR1
MRP2
URATE
1281 1547 677 5871 5564 4369 771 1791 1284 61 1860 5391 738 5387 1670 1908 64 1065 1843 142 4591 5077 4906 612 1078 5659 1409 1429 5893 4548 4790 1475 1712 1846 695 5602 4917 777 1542 289 5579 1737 1380

◀ Fig. 1**a** Anatomical localization of kidney-specific markers, aquaporin 1 (AQP1, proximal tubule marker enriched in cortex) and aquaporin 2 (AQP2, distal and collecting tubules marker enriched in medulla). Uptake and efflux transporters are expressed at the basolateral and apical membrane of kidney epithelial cells in proximal tubules. **b** A flowchart showing the data analysis process for absolute and relative quantification of renal transporters. Based on AQP2/AQP1 ratios, six tissue samples were identified as outliers by Grubbs' test ($p < 0.01$), which were excluded from further analysis. Absolute quantification (method 1) provided an estimate of pmol of drug transporters per gram of tissue, whereas AQP1-normalized relative abundance data (method 2) minimized potential technical variability due to residual contamination from medulla. **c** The six outliers with significantly higher AQP2/AQP1 ratio are shown by red bars. The dotted line parallel to x-axis is the outlier cut-off of 1.98 based on Grubb's test. **d** Heatmap showing significantly lower transporter abundance (pre-AQP1 normalized data, relative abundance per mg membrane protein) in these six outlier samples. The sample IDs and transporters are presented on x- and y-axes, respectively. The six outliers are labeled red on the x-axis and were excluded from further data analysis

the present study. No age-dependent pattern was observed for aquaporin 1 and aquaporin 2 protein levels.

Intra- and Inter-Tissue Protein-Protein Abundance Correlation of Transporter Expression in the Kidney and Liver

Higher intra-tissue protein-protein abundance correlation was observed in the kidney as compared with those in the livers (Fig. 3a, b). Particularly, the protein abundance of renal OAT1-OAT3 showed the highest degree of correlation ($r = 0.87$), which was followed by OATP4C1-MRP2 and MDR1-MRP2 in the kidney ($r \geq 0.8$). In the liver, the highest degree of correlation was observed for MRP2-BSEP ($r = 0.78$), which was followed by MDR1-MRP2 ($r = 0.76$) and BSEP-OATP1B1 ($r = 0.72$).

In paired kidney and liver samples, inter-tissue correlation of transporters expressed in both tissues (*i.e.*, OAT2, MDR1, MRP2, and MATE1) was not observed. A modest correlation was observed between renal OCTN1 with hepatic OCT1 in the paired samples ($r = 0.49$) (Fig. 3c).

DISCUSSION

Very little is known about the developmental changes in renal transporter abundance and activity in children. In this

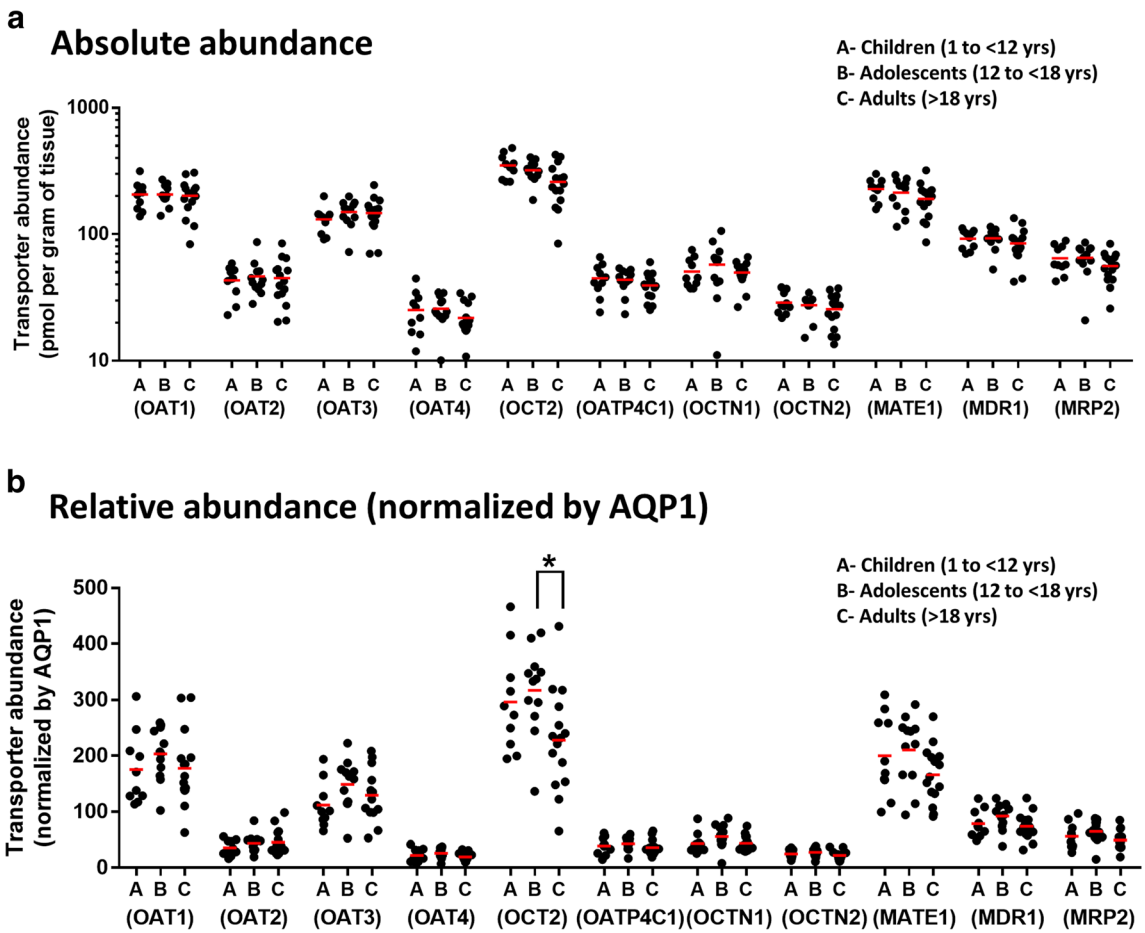


Fig. 2. Protein abundance of renal transporters before (**a**) and after (**b**) normalization by AQP1 in three different age groups, children (1 to < 12 years old), adolescents (12 to < 18 years old), and adults (> 18 years old). Absolute quantification (method 1, **a**) provided an estimate of pmol of drug transporters per gram of tissue, whereas AQP1-normalized relative abundance data (method 2, **b**) minimized potential technical variability due to residual contamination from medulla (Fig. 1). Because of the limited number of infant kidney samples ($n = 1$, < 1 year old), no conclusion could be made whether kidney transporter abundance is different in younger children (< 1 year) *versus* the adults or children age > 1 year. Asterisk (*) represents significant differences between compared groups ($p < 0.05$)

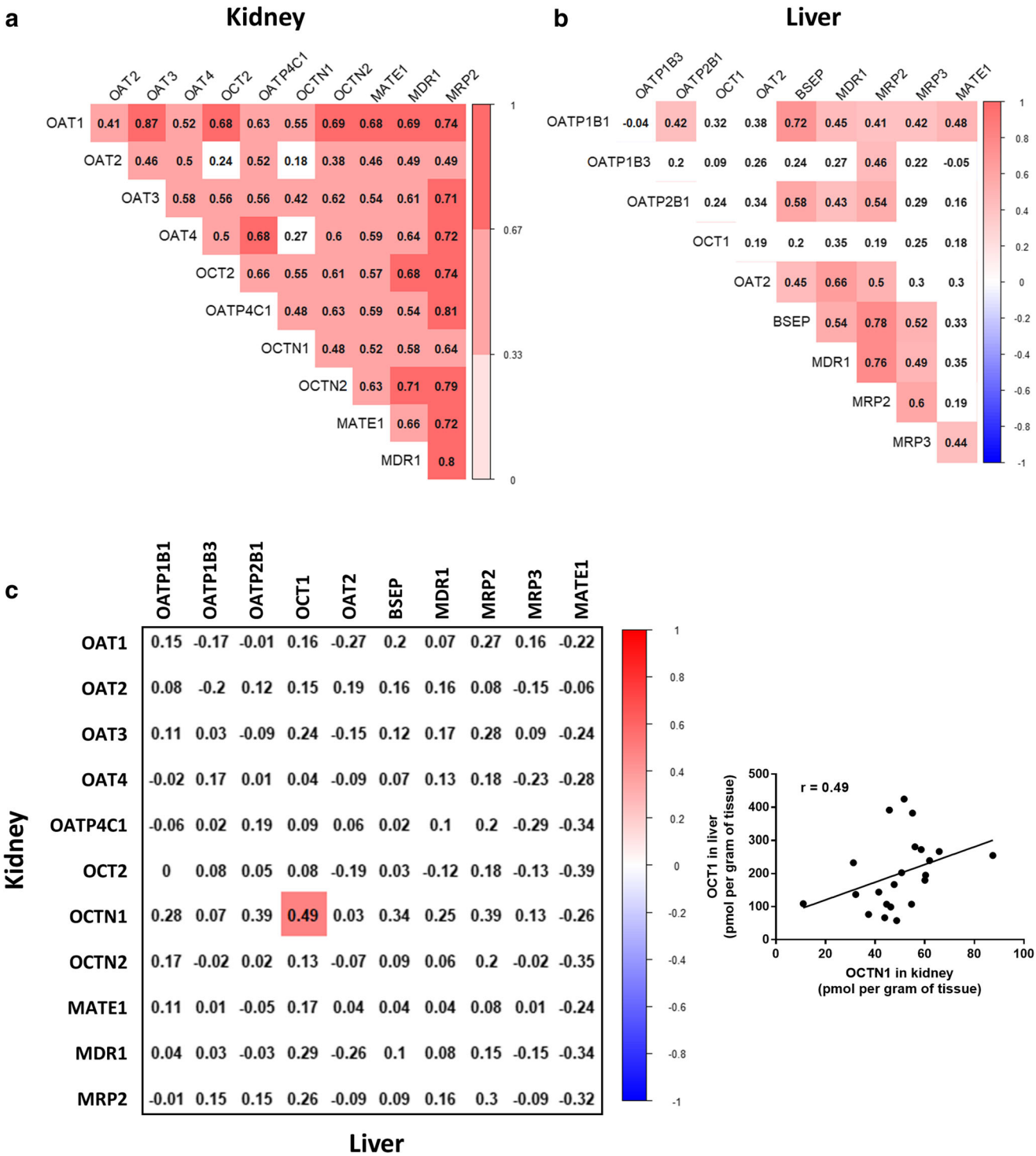


Fig. 3. Protein-protein abundance correlation of transporters in the kidneys ($n = 37$, **a**), the livers ($n = 26$, **b**), and paired kidney-liver samples ($n = 22$, **c**). The Spearman correlation test, with p value < 0.05, was considered as significant (shaded color)

study, we present for the first time the ontogeny of protein expression of renal transporters from children to adults using quantitative LC-MS/MS proteomics. This approach has been used to quantify transporter protein abundances in the human liver (14–16) and in the adult kidney (8).

The kidney is an anatomically complex organ with high cellular heterogeneity, which poses a significant challenge in quantification of renal transporters in archived frozen kidney samples (3). To address this challenge, we used aquaporin proteins, AQP1 and AQP2, as markers of proximal and distal/collecting tubules, respectively. The use of relative AQP2 and AQP1 expression ratio provides a method for excluding kidney tissues with significant medullary contamination prior to data analysis. For example, we excluded six samples, which showed

significantly lower protein abundances of AQP1 and all studied drug transporters. Protein degradation in these six samples was ruled out as the levels of AQP2 were disproportionally higher in these samples. Similarly, Na-K-ATPase, which is expressed in both proximal and distal tubules, was expressed almost equally in all samples (Fig. 1d). To further decrease the technical variability, the renal transporter expression was normalized by AQP1, an approach previously validated by our laboratory (8). This approach can be used to distinguish between biological and technical variability in kidney tissue proteomics analysis.

We did not observe significant age-related changes in the protein abundances of renal transporters (Fig. 2). In contrast to the rat and mouse, the mRNA and/or protein levels of renal transporters (*e.g.*, OAT1, OAT3, OCT2, MRP2, and MRP4) increase with age during postnatal development (17–21). This discrepancy could be a result of species differences and non-availability of samples from younger children and differences in the mRNA and protein levels. Particularly, the kidney development process (*e.g.*, nephrogenesis) in humans is completed by 35-week gestation, whereas in rats, nephrogenesis continues 11 days after birth (17,22). When extrapolating pharmacokinetic data from rats to humans, it is necessary to consider species differences in kidney development and transporter maturation. Further studies are needed with a larger number of pediatric kidney samples from younger age to compare human and rodent ontogeny of these transporters. In addition, we only observed moderate correlation between protein and mRNA expression of renal transporters, including OAT1, OAT3, MATE1, OCTN1, and OCTN2 ($r = 0.46$~0.62), while in the liver, only MRP3 showed high correlation between its mRNA and protein expression ($r = 0.74$) (data not shown). Such poor correlation of mRNA and protein expression has been reported in human liver plasma membrane fraction for several transporters (*i.e.*, MDR1, BSEP, MRP2, BCRP, MATE1, MRP1, NTCP, OCT1, OATP1B3, and OATP2B1) (23). Of the transporters quantified in the kidneys, the protein abundance of OCT2 was the highest, which was consistent with our previous data from the adult kidneys (8). There was no significant association of sex with renal transporter abundance at all age categories (Supplementary Fig. S2). However, this is contrary to sex differences observed in rat, where the OCT2 expression is gender-biased in favor of males perhaps due to species differences in hormonal regulation of transporters (24).

The quantification of renal transporter abundances in both pediatric and the adult kidneys is of critical importance in predicting age-related drug secretion and tissue accumulation in humans. For example, such data in adults have been used for *in vitro* to *in vivo* extrapolation (IVIVE) of metformin (9), which involved integration of the protein abundance of OCT2 in overexpressing cells *versus* kidney tissue into the clearance determined from *in vitro* OCT2-overexpressing cells.

The protein abundance of hepatic MRP3 was significantly lower in children when compared with adults (~ 2-fold) (Supplementary Fig. S3), which is consistent with our previous study on the ontogeny of hepatic transporters (11). Similarly, the protein abundances of OATP1B1, OATP2B1, BSEP, MRP2, and MATE1 were not affected by age, which has been reported before (11,14,16). Since paired samples from younger children (< 1 year old) were not available to us, where OCT1, OATP1B3, and MDR1 abundance have been shown to be lower (11), no age-dependent difference in these transporters was observed in the present study.

Our study has some limitations. First, a larger number of pediatric kidney samples (< 1 year old) will be needed to fully characterize the ontogeny of renal transporters in children. Second, among the paired kidney-liver samples, 15 out of 22 were from adults (> 18 years old). Hence, the observed inter-tissue protein-protein correlations were weighted by adult tissues.

In conclusion, this is the first report of the ontogeny of renal transporters in human pediatric kidney samples. We propose an approach to identify kidney tissues with medullary contamination, which allows better estimation of transporter abundance in kidney cortices for prediction of transporter-mediated drug clearance. Thus, we recommend that the protein abundance of AQP1 and AQP2 should be measured in parallel with renal transporters for sample quality control. Although we were unable to detect age-dependent changes in kidney transporter abundance across ages (> 1 year old), the transporter abundance and protein-protein correlation data could be utilized to predict drug pharmacokinetics using PBPK modeling. Particularly, the abundance data could be used for physiological scaling of *in vitro* clearance data, whereas the correlation data indicate co-regulation and increase predictability of the model. For example, an excellent correlation between OAT1 and OAT3 indicates that OAT1 probe substrate can predict population variability in the OAT3 function (25) similar to CYP3A4 and CYP3A5 *1/*3 correlation equation used in Simcyp (26). Additional investigations involving larger numbers of samples are required to fully characterize ontogeny of renal transporters in children of age < 1 year.

ACKNOWLEDGMENTS

We thank Matthew Karasu from University of Washington for technical assistance with LC-MS/MS sample preparation, and Wendy Wang, Chengpeng Bi, Jeffrey Johnston, and Neil Miller from Children's Mercy Hospital for their contribution to the RNA-seq processing.

FUNDING INFORMATION

This study was supported by UWRAPT (University of Washington Research Affiliate Program on Transporters sponsored by Biogen, Genentech, Gilead, Takeda and Merck & Co., Inc). BP was supported by Eunice Kennedy Shriver National Institute of Child Health and Human Development, NIH grant (R01.HD081299). The National Institute of Child Health and Human Development Brain and Tissue Bank for Developmental Disorders at the University of Maryland is funded by NIH contract HHSN275200900011C, reference no. N01-HD-9-0011.

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