Ontogeny of Small Intestinal Drug Transporters and Metabolizing Enzymes Based on Targeted Quantitative Proteomics

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ABSTRACT

Most drugs are administered to children orally. An information gap remains on the protein abundance of small intestinal drug-metabolizing enzymes (DMEs) and drug transporters (DTs) across the pediatric age range, which hinders precision dosing in children. To explore age-related differences in DMEs and DTs, surgical leftover intestinal tissues related to differences in DMEs and DTs, surgical leftover intestinal tissues from pediatric and adult jejunum and ileum were collected and analyzed by targeted quantitative proteomics for apical sodium–bicarbonate transporter, breast cancer resistance protein (BCRP), monocarboxylate transporter 1 (MCT1), multidrug resistance protein 1 (MDR1), multidrug resistance–associated protein (MRP) 2, MRP3, organic anion–transporting polypeptide 2B1, organic cation transporter 1, peptide transporter 1 (PEPT1), CYP2C19, CYP3A4, CYP3A5, UDP glucuronosyltransferase (UGT) 1A1, UGT1A10, and UGT2B7. Samples from 58 children (48 ileums, 10 jejunums, age range: 8 weeks to 17 years) and 16 adults (8 ileums, 8 jejunums) were analyzed. When comparing age groups, BCRP, MDR1, PEPT1, and UGT1A1 abundance was significantly higher in adult ileum as compared with the pediatric ileum. Our data contribute to the deeper understanding of the ontogeny of small intestinal drug-metabolizing enzymes and drug transporters and shows DME-, DT-, and intestinal location–specific, age-related changes.

SIGNIFICANCE STATEMENT

This is the first study that describes the ontogeny of small intestinal DTs and DMEs in human using liquid chromatography with tandem mass spectrometry–based targeted quantitative proteomics. The current analysis provides a detailed picture about the maturational processes of DT and DME abundances in the human jejunum and ileum. The presented results supply age-related DT and DME abundance data for building more accurate PBPK models that serve to support safer and more efficient drug dosing regimens for the pediatric population.

Introduction

Oral drug delivery in the pediatric population is more challenging than in adults. It is the preferred dosing route for patients of all ages but adds a layer of complexity to the determination of pediatric dosing regimens that are often extrapolated from adult values based on body weight or surface area (Ritschel, 1980; Batchelor and Marriott, 2015; Sawrey et al., 2019). These methods do not take developmental changes of absorption, distribution, metabolism, and excretion (ADME) pathways into account, resulting in age-related variation in exposure that increase the risk of toxicity or therapy failure (Weiss et al., 1960; Ferro, 2015). Although our understanding of ontogeny of ADME processes has greatly increased over recent years, the ontogeny of intestinal drug transporters (DTs) and drug-metabolizing enzymes (DMEs) remains unsolved. Drug transporters are comprised of ATP-binding cassette (ABC) and solute carrier (SLC) families that reside in the luminal and basolateral plasma membranes of enterocytes. In contrast, drug-metabolizing enzymes are present in the cytosol or localize to the membranes of the endoplasmic reticulum. These protein families play a key role in

Abbreviations: ABC, ATP-binding cassette; ADME, absorption, distribution, metabolism, and excretion; ASBT, apical sodium–bicarbonate transporter; BCRP, breast cancer resistance protein; BLQ, below limit of quantitation; DME, drug-metabolizing enzyme; DT, drug transporter; FA, formic acid; FDR, false discovery rate; IHC, immunohistochemistry; LC-MS/MS, liquid chromatography with tandem mass spectrometry; LLOQ, lower limit of quantitation; MCT1, monocarboxylate transporter 1; MDR1, multidrug resistance protein 1; MRP, multidrug resistance–associated protein; OATP2B1, organic anion–transporting polypeptide 2B1; OCT1, organic cation transporter 1; PEPT1, peptide transporter 1; PI, protease inhibitor; PNA, postnatal age; SIL, stable isotope-labeled; SLC, solute carrier; TQP, targeted quantitative proteomics; UGT, UDP glucuronosyltransferase.
Ontogeny of Small Intestinal Enzymes and Transporters

Determining oral drug bioavailability (Giacomini et al., 2010; Xie et al., 2016; Zamek-Gliszczynski et al., 2018). Recently, the age-dependent variation in hepatic and renal abundances of DTs has been investigated by our group and others using proteomics (Prasad et al., 2016; Bhatt et al., 2017, 2019; Boberg et al., 2017; van Groen et al., 2018; Cheung et al., 2019; Ladumor et al., 2019; Li et al., 2019). These data imply that the abundance of intestinal DMEs and DTs follow specific developmental patterns. Similarly, for the intestine, available gene expression and semiquantitative protein data indicate DT- and DME-specific variation as a function of age. For example, multidrug resistance protein 1 (MRD1) protein was detected from the 12th week of gestational age by immunohistochemistry (IHC). Interestingly, postnatal MRD1 mRNA expression levels were similar in neonates and adults (Koneczena et al., 2011; Mootij et al., 2014). In contrast, Western blot analysis showed that CYP3A4 protein levels in fetal and neonatal duodenum are significantly lower as compared with other pediatric age groups (Johnson et al., 2001). Unfortunately, the methods used to obtain this information have several limitations. Firstly, IHC is suboptimal for quantitation of protein expression. Secondly, mRNA expression may not reflect protein content and enzymatic activity in intestinal tissue because of translational and post-translational variations (Couto et al., 2019). Finally, Western blot requires significant amounts of tissue to measure protein content, which is especially challenging in the pediatric population, in which tissue is scarce.

Researchers have therefore looked to apply more advanced quantititative approaches, such as targetted quantitative proteomics (TQP), to determine ADME protein levels in animal and adult human intestinal tissues (Kuno et al., 2019; Couto et al., 2020). Unlike Western blot and quantitative polymerase chain reaction, TQP does not require large tissue amounts. Furthermore, TQP allows for multiplexing, making this method a viable technique to maximize the amount of information that can be obtained from pediatric tissue samples that are often small and scarce (Yu et al., 2020).

Based on the available pediatric data on ontogeny of ADME protein levels, we hypothesized that small intestinal DTs and DMEs would show significant age-related, intestinal region-specific, and protein-specific maturation. Using TQP, we aimed to assess the abundance of clinically relevant DTs and DMEs in jejunal and ileal samples across the pediatric age range and compare them to adult values.

Materials and Methods

Chemicals and Reagents. EDTA, sucrose, histidine, dithiothreitol, iodocetamide, triethylammonium bicarbonate buffer, formic acid (FA), and trifluoroacetic acid were obtained from Sigma-Aldrich (Poole, UK). Rapigest was obtained from Waters Corporation (Milford, MA). Sequencing grade modified trypsin was purchased from Promega (Madison, WI). Protease inhibitor (PI) (P8340) was obtained from Sigma-Aldrich (Poole, UK). Pierce Rapid Gold BCA assay kit and PBS without Ca2+ and Mg2+ were obtained from Thermo Fisher (Paisley, UK), whereas stable isotope-labeled (SIL) internal standards of analytical grade with a purity ≥95% were purchased from Thermo Fisher (Rockford, IL). CryoPREP (CP02 Covaris) and Tissue Tube TTI were obtained from Covaris (Woburn, MA).

Tissue Samples. Pediatric small intestinal tissues were obtained as leftover material from intestinal surgeries at Erasmus MC Sophia Children’s Hospital (Rotterdam, The Netherlands). These jejunal and ileal tissues were assessed for exact anatomic localization by the surgeon. To minimize the risk of tissue degradation, collection was done as follows: All tissues were kept on ice (0 °C) until snap-frozen within 30 minutes in precooled isopentane (~80 °C) except for one sample that was conserved after 90 minutes (Mager et al., 2007). Additionally, ileal tissues were obtained with a similar protocol at Radboud UMC (Nijmegen, Netherlands). These tissues were collected and stored in carbogenated Krebs-Ringer buffer (0 °C) until snap-frozen in liquid nitrogen within 2 hours after excision (Polentarutti et al., 1999). The pediatric tissue collection protocol was evaluated by the Research Ethics Boards of Radboud UMC (CMO number: 2017-3958) and Erasmus MC (MEC 2012-280). As only leftover tissue and electronic health record data were collected, the need for formal Research Ethics Boards approval was waived according to the Dutch Law on Human Research. Written parental and patient-informed consent forms were obtained ahead of the surgical procedures. Detailed characteristics of the pediatric patients are provided in Supplemental Table 1. Because of privacy and anonymization regulations, patient information except age and underlying diagnosis were not available to us. Leftover adult jejunal tissues from Whipple procedures and terminal ileal tissues from (hemi)colectomies were collected anonymously at the Radboud UMC (Nijmegen, The Netherlands). As no patient-related information was accessed, no ethical approval and informed consent was necessary based on the Dutch Law on Human Research. Adult intestinal tissues were handled in the same way as described for the pediatric tissues collected at the Radboud UMC.

Sample Preparation. All procedures were performed with precooled reagents and instruments at 4 °C. Snap-frozen intestinal tissue samples (~150 mg) were crushed using a cryoPREP CP02 (Covaris, Woburn, MA). The crushed tissue powder was suspended in 1 ml of homogenizing buffer (solution C) (pH 7.4) consisting of PBS, 0.5 mM EDTA, 0.25 M sucrose, 5 mM histidine, and PI cocktail (0.1% v/v) (Hatley et al., 2017). The suspended samples were homogenized using a 10-ml Fisherbrand Potter-Elvehjem Homogenizer (Fisher Scientific, Loughborough, UK) with 10 passes (1250 rpm) while keeping the sample on ice. The homogenate was treated with an ultrasonic probe (Quonica Llc, New- town, CT) while on ice for two 10-second bursts at 100% amplitude with a 30-second resting interval. It was then transferred into a 1.5-ml ultra-centrifuge tube (Beckman Coulter Life Sciences, Indianapolis, IN) and centrifuged at 100,000 × g using a TLA-55 fixed angle rotor ultracentrifuge (Beckman Coulter Life Sciences, Indianapolis, IN). The supernatant was collected and stored at ~80 °C. The pellet was weighed prior to resuspending in 500 μl of solution C in an Eppendorf tube and then rehomogenized with the ultrasonic probe for two 10-second bursts at 50% amplitude with a 30-second resting interval while on ice. The total protein concentration of the postcentrifugation resuspended pellet fraction (crude membrane) was determined with Pierce Rapid Gold BCA assay kit.

Approximately 500 μg of the resuspended pellet fraction was taken, and the volumes for each sample were adjusted equally with 10 mM triethylammonium bicarbonate. After adding 10 μl of horse myoglobin (0.4 mg/ml) and 50 μl of 0.2% RapiGest, the samples were reduced with 10 mM dithiothreitol (final concentration) at 60 °C for 30 minutes and alkylated with 15 mM iodoacetamide (final concentration) in the dark at room temperature for 30 minutes. The samples were then spiked with 6.6 mM (final concentration) SIL internal standard, and proteolytic digestion was performed using sequencing grade modified trypsin (enzyme/substrate ratio: 1:25) at 37 °C for 4 hours. After this time, the same amount of sequencing grade modified trypsin was added to the samples and left to incubate for another 12 hours at 37 °C. The reaction was quenched with 0.5% trifluoroacetic acid (final concentration) and incubated for 45 minutes at 37 °C to cleave RapiGest. Finally, the digests were centrifuged at 16,000 × g for 15 minutes, and the clear supernatant was collected. The samples were diluted (1:1) with 0.1% FA, and 50 μg of protein was loaded on column for liquid chromatography with tandem mass spectrometry (LC-MS/MS) analysis.

LC-MS/MS Analysis. Multiple reaction monitoring analysis was performed using a 6500 QTRAP (ABSciex UK Limited, Warrington, UK) coupled to a Shimadzu LC (LC-30A Nexera) UHPLC system (Shimadzu, Wood Dale, IL). Fifty microliters of diluted digests was injected into the LC-MS/MS system. Chromatographic separation was achieved by using a reverse phase C18 column (UPLC PEPTIDE XB-C18 LC column (100 × 2.1 mm, 1.7 μm, Phenomenex, Torrance, CA)) and gradient elution at a flow rate of 500 μl/min. A linear gradient of 5%–40% B (98% acetonitrile in 0.1% FA) in 22.5 minutes followed by a 4-minute wash with 100% B was employed. The column oven was maintained at 50 °C, and autosampler was maintained at 8 °C. All experiments were performed in positive electrospray ionization mode. The ion source temperature and capillary voltages were kept constant at 500 °C and 5.5 kV, respectively.

The small intestinal transporters and enzymes [ABT, BCRP, CYP3C19, CYP3A4, CYP3A5, MCT1, MRD1, multidrug--resistance--associated--protein (MRP) 2, MRP3, organic anion--transporting polypeptide 2B1 (OATP2B1), OCT1, peptide transporter 1 (PEPT1), UDP glucuronosyltransferase (UGT) 1A1, UGT1A10, UGT2B7, and villin-1] were selected based on their clinical relevance and prevalence (Zamek-Gliszczynski et al., 2018). A single multiple reaction monitoring acquisition method was used for all the transporters and
enzymes (Supplemental Table 2). Initially, SIL peptides were analyzed using a Q-Exactive Plus (ThermoScientific, Bremen, Germany) on data-dependent acquisition mode. Subsequently, Skyline 20.1.0.76 software (University of Washington, Seattle, WA) was used to select the top three high-intensity transitions for each peptide. These transitions were monitored simultaneously for identification and quantification. Each selected peptide was unique to the protein of interest and followed the in-silico peptide criteria defined by Kamie et al. (2008). Two peptides per protein were analyzed. Declusterings potentials and collision energies were manually optimized for every peptide by infusion tuning. The most intense transition of the peptide was used for quantitation (Griess et al., 2013; Drozdzik et al., 2018). SIL peptides were used as a single point calibrator for absolute quantification. Prior to sample analysis, calibration curve linearity was checked for each peptide, and based on this analysis, the appropriate amount of SIL was spiked into the sample. Lower limit of quantitation (LLOQ) was also determined during this test (Supplemental Table 2). Peak identification, integration, and quantification were performed using Analyst MultiQuant 3.3 software.

Data Analysis and Statistics. Protein abundance data were measured in pmol/mg membrane protein. For samples below limit of quantitation (BLQ) values, data imputation was performed by adding LLOQ/2 (pmol/mg membrane protein) values to the non-normalized dataset to replace BLQ datapoints (Supplemental Table 4). Sample heterogeneity made normalization of the protein abundance values necessary, and villin-1 was selected as a constitutively expressed enterocyte marker (Zhang et al., 2020). Two different normalization calculations were performed depending on the analysis. In the first approach, protein abundance values (pmol/mg membrane protein) were multiplied by the ratio of villin-1 protein abundance in the sample and the average villin-1 protein abundance in the studied tissue (eq. 1; Figs. 1 and 2).

\[
\text{protein abundance in sample} \times \frac{\text{villin} \, -1 \, \text{protein abundance}}{\text{average villin} \, -1 \, \text{protein abundance}}
\]  

(1)

In the second approach, protein abundance values (pmol/mg membrane protein) were divided by the villin-1 abundance (pmol/mg membrane protein) and \(\log_2\)-transformed to normalize the values (eq. 2; Supplemental Table 4; Tables 1 and 2).

\[
\log_2 \left( \frac{\text{protein abundance in sample}}{\text{villin} \, -1 \, \text{protein abundance}} \right)
\]  

(2)

Relationship between age and protein abundance. Age groups were defined for jejunum (0–2 years, 2–18 years, adults) and ileum (0–2 years, 2–12 years, 12–18 years, adults). To explore differences between age groups, for the villin-1 corrected protein abundances with the imputed values included, the Kruskal-Wallis test was performed. Significance values were adjusted using Bonferroni correction for multiple comparisons among age groups. In the following step, Bonferroni correction for the number of different proteins was implemented by adjusting the significance threshold to \(P < 0.0033\) (Fig. 1). The same analysis was performed on the non-normalized dataset also (Supplemental Fig. 1).

Additionally, the relationship between postnatal age (PNA) and the villin-1–corrected protein abundances was determined by calculating the Spearman’s correlation coefficient. False discovery rate (FDR) correction was used to account for multiple comparisons. All calculated values are shown in Table 1; only comparisons with \(P < 0.0033\) were considered relevant.

Correlations between proteins in the pediatric ileum were calculated using Spearman’s correlation and shown in Table 2 if they had an adjusted \(P\) value \(< 0.000476\) after Bonferroni correction. Non-normalized pediatric ileal abundance data were also interrogated (Supplemental Table 3) with the same statistical probe and stringency.

Statistical analyses were performed, and figures were generated with Graphpad Prism version 5.0.3, IBM SPSS Statistics version 25, and R version 4.0.3.

Results

Tissue Collection. Small intestinal tissue samples from 58 pediatric (jejunum, jejun-ileal junction, proximal and distal ileum, terminal ileum) and 16 adult patients (proximal jejunum, terminal ileum) were available for the study. At Erasmus MC 36 ileum and 10 jejunum samples from pediatric patients aged between 0 weeks and 16 years were collected. Left-over ileal tissues from 12 patients aged between 8 weeks and 17 years were obtained at Radboud UMC. Detailed description of the pediatric samples regarding patient PNA, gestational age, exact tissue origin, underlying disease, and surgical intervention is summarized in Supplemental Table 1. All adult tissues (8 jejunal and 8 ileal samples) were collected at Radboud UMC without any accompanying clinical data.

Protein Expression. In total, 82% of the samples yielded values above the LLOQ. Protein quantitation in 21% of pediatric samples and 8% for adults was BLQ. OATP2B1, MDR1, PEPT1, and villin-1 were detected in all samples. The number of samples BLQ for the other proteins are shown by age group in Supplemental Table 4. High interpatient variability in abundance was detected for most proteins (Figs. 1 and 2; Supplemental Figs. 1–3; Supplemental Table 4). Villin-1 abundance was not significantly different between the age groups.

Relationship of Villin-Normalized Protein Abundance with Age—Analysis by Age Groups. In ileum (\(n = 56\), BCRP protein levels were significantly higher in adults (102.3 ± 37.19 pmol/mg) than in the 0- to 2-year age group (31.75 ± 33.84 pmol/mg; \(P < 0.0033\)) and in the 12- to 18-year age group (28.72 ± 17.18 pmol/mg; \(P < 0.0033\)) (Fig. 1). MDR1 was significantly higher in adults (154.3 ± 53.78 pmol/mg) than in the 0- to 2-year age group (43.57 ± 21.76 pmol/mg; \(P < 0.00067\)). PEPT1 protein abundance was elevated in adults (156.0 ± 42.51 pmol/mg) when compared with the 0- to 2-year age group (58.34 ± 31.73 pmol/mg; \(P < 0.00067\)). UGT1A1 protein was higher in adults (150.3 ± 83.93 pmol/mg) than in the 0- to 2-year age group (11.73 ± 17.91 pmol/mg; \(P < 0.000067\)). In jejunum (\(n = 18\)), BCRP protein levels were higher in adults (92.33 ± 16.55 pmol/mg) than in the 0- to 2-year age group (31.98 ± 11.11 pmol/mg; \(P < 0.0033\)). MRP2 was significantly higher in adults (71.45 ± 24.11 pmol/mg) than in the youngest age group (15.39 ± 10.48 pmol/mg; \(P < 0.0033\)). UGT1A1 protein abundance increased with age: the abundance was higher in adults (441.6 ± 227.5 pmol/mg) than in young infants (18.41 ± 13.05; \(P < 0.0033\)). CYP3A4 protein levels were elevated in adults (563.6 ± 588.1 pmol/mg) compared with the youngest age group (75.80 ± 64.05 pmol/mg; \(P < 0.0033\)).

Age Effect on Villin-Normalized Protein Abundance Measured on a Continuous Scale. In the ileum (\(n = 48\), a positive correlation with PNA was observed for ASBT, MDR1, PEPT1, and UGT1A1, and a negative correlation was observed for MCT1, CYP2C19, and UGT2B7 (Fig. 2; Table 1). After FDR correction, a positive correlation with PNA remained for PEPT1 and UGT1A1, and a negative correlation remained for MCT1 and UGT2B7. In the jejunum only BCRP increased significantly with PNA in the pediatric jejunum (\(n = 10\)).

Interprotein Abundance Correlations. Correlations between protein abundances in the pediatric ileum (\(n = 48\)) are summarized in Table 2. In general, DTs and DMEs showed a positive correlation with each other at a significance level of \(P < 0.000476\), whereas negative correlation was not detected. No correlation was found between BCRP, MRP3, UGT1A1, UGT2B7, CYP3A4, and CYP3A5 and any of the other proteins investigated.

Discussion

We present from a large cohort of pediatric and adult patients the intestinal protein abundance data of DMEs and DTs that are relevant to intestinal drug metabolism and transport. To our knowledge, this is the first report demonstrating the application of TQP to quantify the DMEs and DTs in the intestine of the pediatric population. With our newly developed TQP method, we were able to quantify all proteins in both
Fig. 1. Relative protein abundances of ABC and SLC transporters, cytochrome P450, and UGT enzymes in the jejunum and ileum of pediatric and adult patients compared among age groups. Data are expressed as pmol/mg membrane protein after normalization for average villin-1 protein abundance (see data analysis section in Methods and Materials). Closed symbols provide a single value determined in singlicate, bars represent medians; empty symbols represent imputed values. Adjusted significance: *P < 0.0033, **P < 0.00067, ***P < 0.000067
Fig. 2. Relative protein abundances of ABC and SLC transporters, cytochrome P450, and UGT enzymes in the jejunum and ileum of pediatric patients plotted on a continuous age scale. Data are expressed as pmol/mg membrane protein after normalization for average villin-1 protein abundance (see data analysis section in Methods and Materials). Lines are linear regressions fitted on the data. Circles and solid line: jejunal samples; triangles and dotted line: ileal samples.
Correlation of small intestinal protein abundances of DTs and DMEs with age in pediatric patients

<table>
<thead>
<tr>
<th>Protein/Enzyme</th>
<th>Jejunum (Corrected)</th>
<th>Ileum (Corrected)</th>
<th>Jejunum (Without Correction for Villin)</th>
<th>Ileum (Without Correction for Villin)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BCRP</td>
<td>0.6044</td>
<td>0.6414</td>
<td>0.3769</td>
<td>0.0789</td>
</tr>
<tr>
<td>ASBT</td>
<td>0.3269</td>
<td>0.7124</td>
<td>0.2492</td>
<td>0.2492</td>
</tr>
<tr>
<td>MDR1</td>
<td>0.304 ns</td>
<td>0.2067 ns</td>
<td>0.304 ns</td>
<td>0.2067 ns</td>
</tr>
<tr>
<td>PEPT1</td>
<td>0.4377 ns</td>
<td>0.0790 ns</td>
<td>0.4377 ns</td>
<td>0.0790 ns</td>
</tr>
<tr>
<td>UGT1A1</td>
<td>0.4377 ns</td>
<td>0.4377 ns</td>
<td>0.4377 ns</td>
<td>0.4377 ns</td>
</tr>
<tr>
<td>UGT2B7</td>
<td>0.2553 ns</td>
<td>0.2492 ns</td>
<td>0.2553 ns</td>
<td>0.2492 ns</td>
</tr>
</tbody>
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**Note:** Spearman correlation coefficient. Values significant at *p < 0.05.*
et al., 2013; Drozdzik et al., 2014, 2019; Akazawa et al., 2018). This observation can be attributed either to varying detection limits of the different methods or to external factors. Diet, for instance, can modulate the expression of transporters. For example, the expression of Slc10a in the jejunum has been shown to be increased after oat-containing diet in mice (Andersson et al., 2017). Rats exhibited similar upregulation of Slc10a2/ASBT by 1α,25-dihydroxyvitamin D₃ (Chen et al., 2006).

To highlight the developmental changes from birth to adulthood, we have included adult samples in our dataset. Also, adult values allow a comparison of the current dataset with literature values, since this is the first time pediatric intestinal protein abundance levels are reported. The recent article by Couto et al. (2020) provides a summarized overview of most recent publications on TQP of adult human intestinal ADME proteins. All research groups applied their own unique methodology, which makes the comparison of the current dataset to the literature data challenging. When comparing adult protein abundance values from our study with literature values, it becomes obvious that the non-normalized values are higher in comparison with data obtained by other research groups. The underlying reasons can include the fast preservation of the collected tissue samples, the use of the membrane fractionation, application of PI cocktail from the beginning of the sample preparation, thorough sonication, or extensive digestion. Consistency in the digestion was addressed by spiking equal amounts of horse myoglobin into all the samples prior to digestion (Supplemental Fig. 4). Addition of heavy peptides was used also as a quality-control measure to correct for any discrepancies that might occur during sample clean-up and with the LC-MS/MS system.

Protein abundance data in the current study are presented in two ways: 1) relative to villin-1, a constitutively expressed age-independent marker for normalizing for enterocyte levels in intestinal samples, or 2) without normalization. The advantage of normalization is to overcome the issue of uneven sampling in a heterogenous, multilayered tissue like the small intestine. Since in the intestinal tract villin-1 is only expressed in the enterocytes, it is a suitable normalization factor for the number of enterocytes (West et al., 1988). Additionally, it has previously been shown that villin-1 is stable across age groups, including the fetal and neonatal groups (Johnson et al., 2001). Also, according to our own measurements, villin-1 abundance is stable across age groups. However, most published studies describe uncorrected data, and only few research groups applied villin-1 normalization (Vaessen et al., 2017; Zhang et al., 2020). We have chosen to present our data in both ways in an attempt to make cross-comparison with literature results feasible. A relatively large proportion of pediatric samples had abundance values below the LLOQ. The number of proteins that fall below the LLOQs per tissue correlated with villin-1 abundance (unpublished data). Consequently, LLOQ data may be explained by low villin-1 abundances. Because villin-1 abundance is directly proportional to the number of enterocytes in the sample, we hypothesized that the average yield of all proteins was similarly affected, explaining our finding of relatively frequent protein abundances below the LLOQ, and fortified our assumption that normalization was necessary for the interpretation of the data. We used surrogate values to address the issue and increase statistical power. As we are aware that imputing missing with low values may bias the results, we conducted our statistical analyses with or without including the LLOQ/2 values. Table 1 compares the different outcomes of these calculations. Leaving out BLQ values would be inappropriate because these would then be “missing not at random,” which is poison to most statistical methods.

Although no striking differences were found in most protein abundances, it does not mean that there is no age effect on the intestinal metabolism and pharmacokinetics of drugs. There are multiple reasons that back this observation: 1) The effect is influenced/masked by

<table>
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<th>TABLE 2</th>
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<tr>
<td><strong>Correlation of villin-1 normalized protein abundances of DTs and DMEs with each other in the pediatric ileum.</strong></td>
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<tr>
<td></td>
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<tr>
<td>BCRP</td>
</tr>
<tr>
<td>MRP2</td>
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<td>MIP3</td>
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<td>MRCP</td>
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<td>MCT1</td>
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<td>MCT2</td>
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<tr>
<td>OATP2B1</td>
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<tr>
<td>MDR1</td>
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<tr>
<td>MDR2</td>
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<td>OAT1</td>
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<tr>
<td>OCT1</td>
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<tr>
<td>UGT1A1</td>
</tr>
<tr>
<td>UGT1A10</td>
</tr>
<tr>
<td>CYP2C19</td>
</tr>
<tr>
<td>UGT2B7</td>
</tr>
<tr>
<td>CYP3A4</td>
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*Spearman r-values are shown if *P* < 0.000476.
medications and food the patients received around the time of sampling. These drugs might change the abundance of DTs and DMEs. Children admitted to hospitals usually have serious health conditions and require medication(s). Disease can also affect DT and DME quantities, but collecting tissues from healthy children is not possible. To reduce the impact of disease we only collected tissue from nondiseased areas. For the stoma tissues, we used the nondiseased areas of the tissue as close to the excision site as possible to avoid using externally exposed tissue. 2) The effect of age is not linear but follows a certain developmental trajectory. This could be solved by including higher number of patients divided into more age groups, but sample collection is a main challenge. 3) Despite our efforts to collect leftover tissue material from all age groups, the neonatal age group was underrepresented in our study population. 4) Genetic polymorphisms may affect gene expression and, ultimately, the function of DTs and DMEs. These investigations would be highly valuable but also introduce new variables. In this experimental setup, drawing proper conclusions would require a larger population. 5) Furthermore, TQFP quantitates the protein of interest but gathers no information about its activity. 6) Posttranslational modifications could account for discrepancies when the abundance of a protein does not correlate with its activity. 7) DME and DT activity measurements were out of the scope of the current work, but future studies can bridge this gap in the ontogeny of DTs and DMEs.

The possible implications of our findings include the therapeutic dosing of drugs for pediatric patients. In the current clinical practice, accurate dosing is imperative for agents metabolized by CYP3A4 and UGT1A1, which were found to exhibit age-dependent maturation. Furthermore, BCRP, MDR1, and MR2P are the main transporters affecting the absorption of the majority of therapeutic substances, thus in the future measuring their abundance could provide the basis for precision drug dosing.

In conclusion, this is the first time that TQFP was applied to study the impact of age on DT and DME protein abundance in small intestinal tissues. In spite of the limitations, we showed that our approach detected significant age-related differences for major, clinically relevant DTs and DMEs. The importance of these changes in relation to intestinal drug absorption remains to be demonstrated through the incorporation of the current dataset into physiology-based pharmacokinetic models. To assess the overall importance of the current findings, it will be critical to simultaneously consider all system parameters that are subject to age (e.g., intestinal surface area, length of the intestinal segments). In itself, the lack of change in the abundance of the majority of DTs and DMEs is already a key finding. It can provide better confidence to physicians to prescribe oral drugs and pharmaceutical companies to develop oral dosing regimens for pediatric patients. Future research should expand the dataset by including more neonates to determine possible changes during the first weeks of extraterrestrial life.

Author Contributions

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