Regional expression levels of drug transporters and metabolizing enzymes along the pig and human intestinal tract and comparison with Caco-2 cells

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Running title: Intestinal expression of transporters and enzymes

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Number of text pages: 20
Number of tables: 2
Number of figures: 4
Number of supplemental data: 3
Number of references: 29
Number of words in the Abstract: 253
Number of words in Introduction: 713
Number of words in Discussion: 1416

Non-standard abbreviations: BCRP, breast cancer resistance protein; BSEP, bile salt export pump; GLUT1, glucose transporter 1; MCT, monocarboxylate transporter; MDR1, multidrug resistance gene; MRP, multidrug resistance-associated protein; NTCP, Na-taurocholate co-transporting polypeptide; OATP, organic anion-transporting polypeptide; OCT, organic cation transporter; PEPT1, peptide transporter 1; CYP, cytochrome P450; HEPES, 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid; KRB, Krebs-Ringer Buffer; SD, standard deviation; SEM, standard error of the mean; DMEM, Dulbecco's Modified Eagle Medium; UGT, Uridine 5'-diphospho-glucuronosyltransferase
ABSTRACT

Intestinal transporter proteins and metabolizing enzymes play a crucial role in the oral absorption of a wide variety of drugs. The aim of the current study was to better characterize available intestinal in vitro models by comparing expression levels of these proteins and enzymes between porcine intestine, human intestine and Caco-2 cells. We therefore determined the absolute protein expression of 19 drug transporters and the mRNA expression of 12 metabolic enzymes along the pig intestinal tract (duodenum, jejunum, ileum; N=4), in human intestine (jejunum; N=9) and Caco-2 cells. Expression of the included transporters and enzymes was in general well comparable between porcine and human intestinal tissue, though BCRP, MCT5, MDR1, MRP1, MRP3 (~2-fold) and OATP4A1 (~6-fold) was higher expressed in pig compared to human jejunum. Alternatively, expression level of relevant transporter proteins (GLUT1, OATP4A1, MRp2, MRP1 and OATP2B1) was significantly higher (3- to 130-fold) in Caco-2 cells compared to human jejunum. Moreover, all examined CYPs showed at least a five-fold lower gene expression in Caco-2 cells compared to human jejunum, with the smallest differences for CYP1A1 and CYP3A5 and the largest difference for CYP3A4 (871-fold higher expression in human jejunum compared to Caco-2 cells). In conclusion, a comprehensive overview is provided of the expression levels of clinically relevant transporter proteins and metabolic enzymes in porcine and human intestinal tissue, and Caco-2 cells, which may assist in deciding upon the most suitable model to further improve our understanding of processes that determine intestinal absorption of compounds.
INTRODUCTION

An accurate prediction of the human intestinal absorption and oral bioavailability early in drug development is essential in the pharmaceutical and nutritional industry, as this co-determines the efficacy and/or toxicity of the active compound. Several in vitro (e.g. Caco-2 cells, HT-29 cells, Ussing chamber) and in silico methods (e.g. GastroPlus and SimCyp) are currently in use to predict human intestinal absorption and subsequently human oral bioavailability of compounds. Initial assays to study intestinal apparent permeability (P_app) of the compound and the effect of efflux transporters (e.g. MDR1, BCRP) on intestinal absorption are often performed with Caco-2 cells, originating from human epithelial colorectal adenocarcinoma cells (Haslam et al., 2011, Yee, 1997, Yazdanian et al., 1998). Whereas the use of Caco-2 cell monolayers as an intestinal barrier model is well-established, and provides a quick and inexpensive screening model, standard Caco-2 cells lack morphological and physiological features of complete intestinal tissue. For instance, standard Caco-2 cultures show differences with complete intestinal tissue with regard to mucus production, passive diffusion, carrier-mediated uptake and excretion, paracellular transport via tight junctions and intestinal metabolism (Rozehnal et al., 2012). However, recent studies in which Caco-2 cells were cultured on porous membranes in a fluidic device with peristaltic movement also demonstrate the formation of villi-like structures and increased metabolizing activity (CYP3A mediated) compared to Caco-2 cells cultured on Transwell membranes under static conditions (Kim and Ingber, 2013), and thereby more closely mimicking the human physiology. We have recently developed the InTESTine™ system (Westerhout et al., 2014), in order to enable to study processes that determine (human) intestinal absorption in a physiological relevant model. In this system ex vivo intestinal tissue (human or porcine) is mounted into a two compartment system, simulating luminal and blood compartments. The inTESTine system is currently optimized to keep the mounted tissue viable for a maximum of 4 hours. It provides some distinct advantages compared to both Caco-2 cell culture systems and the frequently used Ussing chamber model (Rozehnal et al., 2012, Lennemas, 2007). The main advantages compared to Ussing chamber is the higher throughput, using a disposable multi-well setting, and standardized culture conditions using a humidified high oxygen/CO₂ incubator on a rocker platform. The key advantage compared to Caco-2 cells is the presence of complete mucosal tissue, including the presence of different intestinal epithelial cells (enterocytes, Goblet cells, enteroendocrine cells, Paneth cells, and M-cells), the lamina propria including the intraepithelial lymphocytes and other immune cells (Mowat and Agace, 2014). This makes the InTESTine™ model suitable for studies relating to gut
health and gut immune function. Additionally, the presence of a natural mucus barrier in the InTESTine™ system enables the direct combination of biorelevant luminal samples with an in vitro absorption model to better simulate the physiology of the human GI epithelial wall.

The intestinal absorption of compounds across the intestinal epithelium depends on their chemical characteristics, and compounds can be substrates for numerous transporter proteins and metabolizing enzymes. There is, however, limited information available on the absolute expression of transporter proteins and metabolizing enzymes in the currently used models including Caco-2 cells, human and porcine intestinal tissue. Although there is some literature available on the gene expression of drug transporter genes in human intestine and Caco-2 cells (Taipalensuu et al., 2001, Hilgendorf et al., 2007, Englund et al., 2006), mRNA expression levels of transporter proteins are shown not to correlate well with protein abundance levels (Ohtsuki et al., 2012). A lack of absolute expression levels of active transporter proteins and metabolizing enzymes could potentially result in inaccurate classification of the permeability and intestinal absorption of compounds. As pointed out, the various in vitro intestinal models all have their applications and limitations, and it is important to emphasize that there is not one model available that can be used as the golden standard to predict human luminal processes and intestinal absorption. The aim of the current study was therefore to further characterize and compare these potential in vitro intestinal models to determine their feasibility for absorption of compounds. To that end, we quantified the absolute and regional protein expression of several uptake and efflux transporters (BCRP, BSEP, GLUT1, MCT1, MCT5, MDR1, MRP1, MRP2, MRP3, NTCP, OATP4A1, OATP1B1, OATP1B3, OATP2B1, OATP1C1, OCT1, OCT3, OCTN2, PEPT1) at the plasma membrane along the pig intestinal tract and compared these data to expression levels in Caco-2 cells and ex vivo human intestinal tissue. Moreover, mRNA expression profiles of several metabolizing enzymes of important cytochrome P450 and uridine 5’-diphospho-glucuronosyltransferase (UGT) families were studied. In future studies, these abundance data will be integrated in in silico models to be able to better predict human oral bioavailability based on in vitro absorption studies.
MATERIALS & METHODS

Chemicals and reagents

Krebs-Ringer Bicarbonate Buffer and 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid (HEPES) were purchased from Sigma-Aldrich Chemie B.V. (Zwijndrecht, The Netherlands). Dulbecco's Modified Eagle Medium (DMEM), Minimum Essential Medium (MEM), L-glutamine, gentamicin, penicillin, streptomycin and heat-inactivated fetal bovine serum were purchased from Gibco (Paisley, Scotland).

Culturing of Caco-2 cells

The human colon carcinoma cell line Caco-2 was obtained from the German Collection of Microorganisms and Cell cultures (DSMZ ACC 169, Braunschweig, Germany). Caco-2 cells were cultured in HEPES-buffered DMEM containing 4.5 g/L glucose, supplemented with 1% (v/v) MEM non-essential amino acids, 6 mM L-glutamine, 50 mg/L gentamicin and 10% (v/v), heat-inactivated fetal bovine serum. Cells were grown in 75 cm² flasks (Corning-Costar, Cambridge, Massachusetts, United States) at approximately 37°C in a humidified incubator containing a 95% air/5% CO₂ mixture. For protein expression analysis, Caco-2 cells were cultured for 14 days in 75 cm² flasks, which were fully confluent after 3-4 days and when harvested (14 days after seeding) a full epithelial monolayer was formed. Approximately 70 x 10⁶ trypsinized and pelleted cells were used for single plasma membrane isolation (preparation in duplo, n=2). For gene expression analysis, Caco-2 cells were cultured for 21 days on permeable supports (polyester membrane with 0.4 µm pore size, Corning, N.Y., U.S.A.) in 12-well plate with medium replacement every 2-3 days.

Origin of pig and human intestinal tissue

Five healthy domestic pigs (Sus scrofa domesticus, 2 male and 3 female, age 10-14 weeks and bodyweight between 15 and 25 kg) were used for the collection of intestinal tissue. These animals were additionally used for educational purposes at the Utrecht University (Utrecht, The Netherlands) with approval of the local animal welfare office, and in full compliance with the aim to contribute to the reduction, refinement and replacement of animal experiments. Before euthanization, pigs had free access to food and water. Intestinal tissue of domestic pigs was collected only when defined healthy
as judged by a veterinarian. Prior to the isolation of the intestine, 2000 mL Krebs-Ringer Bicarbonate Buffer (containing 10 mM glucose, 25 mM HEPES, 15 mM sodium bicarbonate, 2.5 mM calcium chloride, pH 7.4, and saturated with oxygen using a 95%/5% O₂/CO₂ mixture by gassing for 120 minutes, further indicated as KRB) was divided over different small volume flasks. After sedation, animals were euthanized and segments of duodenal tissue (the first 25 cm from the stomach), jejunal tissue (150 cm from the stomach) and ileal tissue (50 cm from the ileocecal valve) were excised, flushed with ice-cold KRB buffer, stored in ice-cold KRB, transported to the lab, and immediately used for ex vivo preparation. Once in the lab, the intestinal tissue segments were cut into pieces of 10 to 20 cm and cut open longitudinally continuously submerged under ice-cold KRB buffer during further preparation. Then, the upper villus layer of the mucosa was removed with the edge of a glass slide and mucosal cells were collected and quickly stored < -70°C until further processing.

Human jejunum samples derived from 9 individuals (4 female, 5 male) were collected at the University Medical centre of Groningen (UMCG, Groningen, The Netherlands) and were kindly provided by Prof. Dr. G.M.M. Groothuis (University of Groningen, The Netherlands). Collection of redundant intestinal tissue from surgeries (collected as waste material) was approved by the Medical Ethical Committee (MEC) of the UMCG. No clinically relevant or identifiable information from the patients was collected. Intestinal tissue samples were directly snap frozen and stored < -70°C until further processing. The weight of 4 of these tissue samples was sufficient for plasma membrane protein analysis and subsequent quantitative LCMS/MS analysis; the remaining samples were only used for gene expression analysis.

Protein isolation for quantitative LCMS/MS analysis

To determine absolute protein expression levels of BCRP, BSEP, GLUT1, MCT1, MCT5, MDR1, MRP1, MRP2, MRP3, NTCP, OATP4A1, OATP1B1, OATP1B3, OATP2B1, OATP1C1, OCT1, OCT3, OCTN2, PEPT1 and villin at the outer plasma membrane of Caco-2 cells, pig intestinal mucosal tissue (duodenum, jejunum and ileum, n=4 different animals), and human intestinal tissue (jejunum, n=4 different donors), we have followed the protocol of membrane isolation and trypsin digestion as previously described for tissue samples and cell lines (van de Steeg et al., 2013, Bosgra et al., 2014). All samples were processed in duplicate, and a pellet containing 60-75 x 10⁶ Caco-2 cells or approximately 350 mg intestinal mucosal tissue was used for plasma membrane isolation.
intestinal tissue was processed differently, compared to human intestinal tissue, since in case of pig tissue the villi layer was scraped off, whereas in case of human intestinal tissue the complete tissue segment was used (as these samples were snap frozen immediately after section, scraping was not possible anymore). By using villin expression as a marker for epithelial cells (West et al., 1988) we corrected for these differences. Therefore, when comparing human intestine, porcine intestine and Caco-2 cells, only the villin corrected data is presented. After tryptic digestion, peptides were separated on a C18-column (Acquity BEH UPLC column, 2.1 x 100 mm, inner diameter 1.7µm) using a linear gradient of 5-45% mobile phase B (acetonitrile with 0.1% formic acid) during 5 min with a flow of 600µl/min followed by a 2 min wash-out with 100% mobile phase B. Peptides were ionized with electrospray and quantification was performed with a 6500 QTrap (ABSciex) using a scheduled MRM-mode. Cone voltage and collision energy were optimized for each compound individually. Per peptide 3 transitions were chosen (Q3-1, Q3-2, and Q3-3) for quantitation and confirmation (Supplemental Table 1). In case no suitable prototypic peptide could be selected for the human and porcine transporter proteins, two separate peptides were selected and synthesized (Supplemental Table 1). Peptides labelled with 15N and 13C (AQUA peptide) were synthesized (Sigma Aldrich Chemie, Steinheim, Germany) and used as an internal standard for quantification. For each peptide a calibration curve of 0.01 – 50 ng/mL and quality controls were included in every run. Peak identification and quantification was performed using Analyst software version 1.6.

RNA isolation and cDNA synthesis

Total RNA was isolated from flash frozen pig jejunum tissue (5 pigs, ~10 mg of tissue), human jejunum tissue (9 donors, ~10 mg tissue) and from Caco-2 cells after a 21 day differentiation in Transwells (10 independent 12-well incubations from same passage) using the Quick-RNA miniprep kit (Zymo Research, Irvine, CA, USA) which includes a DNase step to ensure complete removal of genomic DNA. Total RNA was transcribed to cDNA using the iScript cDNA synthesis kit (BioRad, Hercules, Ca, U.S.A.) and used directly for qPCR analysis after a 5-fold dilution.

RT-PCR measurements

Primer pairs (see Supplemental Table 2) were designed within 1 exon, allowing absolute copy quantification using a genomic DNA standard curve thus enabling the comparison of results
between different gene targets and between different tissues, \textit{i.e.} pig jejunum, human jejunum and Caco-2 cells. qPCR analysis was performed on a StepOnePlus system (Applied Biosystems, Waltham, MA, USA) using Fast SYBR Green Master mix (Applied Biosystems) according to the protocol provided. Absolute gene copy numbers were calculated using standard curves constructed with human or porcine genomic DNA (Novagen, Merck Millipore, Billerica, MA, USA) as described by Yun et al \cite{Yun2006}. Calculated gene copy numbers were corrected for beta-actin (ACTB) and villin (VIL) copy numbers to correct for material input.

\textit{Data analysis}

One-way ANOVA followed by Tukey’s multiple comparison test was used throughout the study to assess the statistical significance of differences between multiple datasets (GraphPad Prism 4.1 software was used for this). Differences were considered to be statistically significant when $P < 0.05$. 

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RESULTS

**Absolute transporter protein expression along the pig GI tract**

The abundance of a set of transporter proteins along the pig intestinal tract (duodenum, jejunum, and ileum) was determined using quantitative mass spectrometry (Figure 1). We isolated the plasma membrane fractions of duodenum, jejunum and ileum mucosal tissue samples derived from 4 individual domestic pigs. Transporter proteins are functionally active when expressed at the outer plasma membrane. Absolute expression levels of the various transporter proteins ranged between 0.01 and 2 pmol/mg tissue (Figure 1A). The expression of BSEP, NTCP, OATP1B1, OATP1B3, OATP1C1 and OCT3 transporter proteins in pig intestine was below the lower limit of quantification (i.e. ≤ 0.01 ng/mL, equivalent to approximately 0.01 pmol/g tissue) in all tissue samples (Figure 2). Of the detectable transporter proteins, MDR1 and OATP4A1 were most abundantly and almost equally expressed in pig samples of duodenum, jejunum and ileum, followed by MRP3 and BCRP in pig duodenum and ileum, and PEPT1 and BCRP in pig jejunum. Overall, GLUT1, MCT1, MRP2, and MRP3 were among the lowest expressed transporters in pig samples, but their absolute numbers were significantly lower in segments from pig jejunum and ileum compared to duodenum. This could mainly be explained by decreased amounts of epithelial cells in the more distal parts of the gastrointestinal tract, indicated by decreasing amounts of villin, a known epithelial marker (West et al., 1988) (Supplemental Figure 1). After correction for the amount of villin, only the expression of MRP2 remained significantly lower in jejunum and ileum compared to duodenum (Figure 1B–E). Interestingly, an opposite tendency was observed for the transporters MDR1, OATP4A1 and MCT5, with slightly increasing expression levels when comparing the duodenum to the ileum.

**Transporter protein expression in pig and human jejunum in comparison with Caco-2 cells**

Since monolayers of the human intestinal epithelial Caco-2 cells are generally used as an in vitro screening model for human intestinal permeability, we have compared the expression of several transporter proteins in human Caco-2 cells with ex vivo human or pig jejunum tissue samples (Figure 2A). By correcting for the amount of villin (epithelial marker), we enabled direct comparison of transporter protein abundance in human or pig jejunum with Caco-2 cells, and also enabled direct comparison of human and porcine intestinal tissue which were processed slightly differently (mucosal
layer versus whole tissue) resulting in possible differences in the amount of epithelial cells included in the sample (absolute expression of villin in Caco-2 cells compared to pig intestine is presented in Supplemental Figure 1). Villin expression was not significantly different between human and pig jejunum. Comparable to human and pig jejunum, expression of BSEP, NTCP, OATP1B1, OATP1B3, OATP1C1 and OCT3 in Caco-2 cells was below the lower limit of quantification (i.e. \( \leq 0.01 \) ng/mL, comparable to \( \sim 4 \cdot 10^{-6} \) pmol/10E6 cells). Relative expression of GLUT1, OATP4A1, MRP2, MRP1 and OATP2B1 was significantly higher (3- to 130-fold) in Caco-2 cells compared to human jejunum, whereas expression levels of BCRP, MCT1, MCT5, MDR1, MRP3, OCT1, OCTN2 and PEPT1 did not differ significantly between differentiated Caco-2 cells and human or pig jejunum samples. Expression of BCRP, MCT5, MDR1, MRP1, MRP3, and OATP4A1 appeared to be slightly (though significantly) higher in pig jejunum compared to human jejunum (Figure 2A). Absolute expression levels of transporter proteins in pig and human jejunum are presented in Figure 2B.

**Intestinal gene expression of metabolizing enzymes**

To compare gene expression of metabolizing enzymes between human jejunal tissue (n=9), pig jejunal tissue (n=5) and human Caco-2 cells (n=10), we determined gene expression of a selected panel of CYPs and UGTs. Based on data in the literature, the most abundantly expressed and therefore likely the most relevant human CYPs and UGTs were selected, *i.e.* for CYPs: CYP2C9, CYP2J2, CYP3A4 and CYP3A5, and for UGTs: UGT1A1, UGT1A6, UGT1A10, UGT2A3 and UGT2B7 (Bieche et al., 2007, Paine et al., 2006, Pavek and Dvorak, 2008). In addition, four different CYP genes and one UGT gene were included with lower intestinal (protein) expression levels, but with known relevance for human drug metabolism, *i.e.* CYP1A1, CYP2C18, CYP2D6, CYP2E1 and UGT1A8. For every human CYP and UGT gene we attempted to include at least one pig homolog (see Tables 1 and 2). The identified pig homologs shared on average 75% amino acid homology with their human counterparts. Using a genomic DNA calibration curve in every qPCR analysis allowed absolute quantification of copy numbers and therefore the possibility to compare results of different gene targets and between sample types. Relative CYP gene expression (corrected for actin-beta and villin expression) in the three models, *i.e.* human jejunal tissue, human Caco-2 cells and pig jejunal tissue, are presented in Figure 3. As expected for human jejunal tissue, CYP3A enzymes showed the highest expression, whereas CYP1A1 and CYP2E1 were of very low expression. All examined CYPs showed at least a five-fold
lower gene expression in Caco-2 cells compared to ex vivo human jejunal tissue, with the smallest differences for CYP1A1 and CYP3A5 and the largest difference for CYP3A4, i.e. 871-fold higher expression in human jejunum compared to Caco-2 cells. In general, pig CYP homologs in pig jejunum showed expression levels more comparable to human jejunum than to Caco-2 cells. However, whereas the expression level of pig CYP2C42 was comparable to CYP2C enzymes in human jejunum, the expression levels of the pig CYP2C33 and CYP2C49 enzymes were more comparable to expression levels of CYP2C enzymes in Caco-2 cells. **Figure 4** shows the gene expression levels of UGTs. All examined UGTs, except for UGT1A6, showed higher expression levels in human jejunum compared to Caco-2 cells. Expression of pig homologs was for some UGTs comparable to expression in human tissue, i.e. UGT1A10 and to less extent UGT1A1 and UGT1A6, but pig UGT2B enzymes 2B18 and 2B31 were hardly expressed whereas human UGT2B7 showed significant expression both in human ex vivo jejunal tissue and in Caco-2 cells.
DISCUSSION

In this study, we provide a comprehensive data set for the expression of transporter proteins and metabolic enzymes along the pig intestinal tract, and compared these expression profiles with human intestinal tissue and Caco-2 cells. To the best of our knowledge, this is the first study that directly compares expression of a set of active transporter proteins and metabolizing enzymes between porcine intestinal tissue, human intestinal tissue and Caco-2 cells. Here, we also describe for the first time regional differences in expression of a set of 19 transporter proteins in the pig gastrointestinal tract. In this study, we have determined transporter protein expression at the outer plasma membrane of cells, which is the most pure fraction only containing the outer plasma membranes where the transporter proteins are actively expressed. We have previously successfully set-up and used a method for plasma membrane isolation (Bosgra et al., 2014), and used this method in the current study. Various protein isolation protocols have been applied and described in literature for transporter protein abundance using LC-MS/MS, and recent insights show that the loss of proteins during various centrifugations steps is significant (Ohtsuki et al., 2012, Harwood et al., 2014). It is therefore very important to ensure to use the same protein isolation method for comparison between cell lines and (human) tissue levels, and for application in In Vitro-In Vivo Extrapolation (IVIVE). In the current study we have also used villin expression to normalize for the amount of epithelial cells in order to enable to directly compare between tissue types and cell lines.

Regional differences in expression of transporter proteins in human intestinal tissue have been observed before by Western blot analysis for single transporter proteins (Englund et al., 2006, Meier et al., 2007). Interestingly, a recent paper describes the protein abundance of relevant drug transporters in differential regions of the human gastrointestinal tract (Drozdik et al., 2014), using a crude membrane extraction procedure. Since this crude membrane preparation differs from the plasma membrane preparation used in the current study, direct and quantitative comparison between the studies is difficult. Nevertheless, Drozdzik and co-workers observed regional-dependent differences in the level of protein expression for MDR1, which appeared to be higher towards the more distal parts of the small intestine. A similar pattern was observed in the current study for MDR1 in pig intestinal tissue, as was the case for OATP4A1 and MCT5 (these latter 2 proteins were not included in the study by Drozdik). For all other transporter proteins no regional differences in absolute expression levels were detected in the current study. Using a slightly different detection method (QconCat, in which isotope
labelled peptides are generated by proteolytic digestion of an artificial protein constructed within *E. coli* and a crude membrane isolation method, Harwood et al has recently described the expression of MDR1, BCRP, and MRP2 in human jejunum and ileum tissue samples (Harwood et al., 2015). Relative differences in abundance between these 3 transporter proteins confirmed lowest expression of MRP2 compared to MDR1 and BCRP in human jejunum (and ileum), which we also found in our data set for human jejunum. Moreover, the same trend of lowest MRP2 expression compared to MDR1 and BCRP was observed in pig intestinal tissue (duodenum, jejunum, and ileum), indicating good similarity between human and pig intestinal tissue.

Since monolayers of Caco-2 cells are generally used as an *in vitro* screening model for assessment of human intestinal permeability, we compared expression of a set of transporter proteins in human small intestinal tissue with Caco-2 cells. In a recent paper by Harwood et al, they describe a cross-laboratory study between 2 laboratories where they have looked at expression of MDR1 and BCRP in human jejunum and Caco-2 cells (Harwood et al., 2016). Both laboratories observed 2-fold higher absolute expression of MDR1 in Caco-2 cells compared to human jejunum, and 1.5 to 2-fold decreased expression of BCRP in Caco-2 cells compared to human jejunum. Similar trends for MDR1 and BCRP in comparing Caco-2 cells with human jejunum were observed in the current study (Figure 2A). Compared to the data published by Drozdzik et al, Pept1 expression in the current dataset of human and porcine intestinal tissue is relatively low, which could possibly be explained by differences in sample preparation (Drozdzik et al., 2014). Remarkably, we found that expression of GLUT1, which is generally of very low expression in vivo in the gut epithelium, was more than 130-fold higher in Caco-2 cells compared to human jejunum tissue. This may be caused by the colorectal adenocarcinoma origin of Caco-2 cells and/or the fact that the cells are cultured in glucose-rich medium. Also OATP4A1, MRP1, MRP2 and OATP2B1 were significantly higher expressed in Caco-2 cells compared to human jejunum, demonstrating the differences between Caco-2 cells and human intestinal tissue. It should be noted however, that for plasma membrane protein isolation the Caco-2 cells were cultured in culture flasks rather than filter inserts. Though cells were grown to full confluency and were cultured for 14 days to form a differentiated epithelial monolayer in the culture flasks, some differences with respect to differentiation of the Caco-2 cells may need to be taken into account compared to Caco-2 cells cultured on filter inserts.
In order to determine metabolic enzyme expression in the three models we examined gene expression of a panel of CYPs and UGTs. Gene expression levels of metabolic enzymes are generally considered to correlate well with protein levels of the respective enzymes. For example, human intestinal gene expression levels of different CYPs as determined in this study and previously reported by Biechi et al (Bieche et al., 2007) correlate well with the determined protein levels of these CYPs as reported by Paine et al (Paine et al., 2006). Overall, the distribution of CYP isoform gene expression in human jejunum are in good agreement with these previous studies, with the rank order of enzyme expression ranging from highest to lowest; CYP3A>CYP2C9>CYP2C18>CYP2J2>CYP2D6>CYP1A1>CYP2E1. In addition, the UGT gene expression levels we measured in human jejunum are in agreement with data previously reported by Siissalo et al (Siissalo et al., 2008) showing substantially lower expression of UGT1A8 compared to the other five tested UGTs. Our finding that Caco-2 cells have strongly reduced expression levels of all but one tested CYP and UGT enzymes compared to ex vivo human tissue is in accordance with expectations based on literature data (Siissalo et al., 2008, Zhang et al., 2011, Sun et al., 2002). Indeed, only the gene expression of UGT1A6 was found to be higher in Caco-2 cells compared to human jejunum, which confirms findings by Siissalo et al (Siissalo et al., 2008). Comparing human CYPs and UGTs with pig homologs is difficult because interspecies homology is not always evident. Some human CYPs and UGTs have pig-specific counterparts, including the pig homologs for CYP1A1, CYP2D6, CYP2E1 and UGT1A6, which also show remarkably similar expression levels in both species. For the other human CYP and UGT genes it is more difficult to identify specific pig counterparts, for example the human CYP2C9 and CYP2C18 enzymes for which we found three CYP2C homologs in pig. Whereas CYP2C42 has comparable expression levels in pig tissue compared to human tissue the other two pig-specific CYP2C isoforms show very low expression in pig jejunum, as also previously shown (Puccinelli et al., 2010). Taken together, overall CYP and UGT enzymes in pig jejunum are expressed at comparable levels as in human jejunal tissue and substantially higher than in Caco-2 cells, which would make ex vivo pig tissue a better model than Caco-2 cells to determine the effect of intestinal wall metabolism on oral absorption of compounds. However, substantial differences in substrate specificity between human and pig CYPs and UGTs have been observed and need to be taken into account when using pig intestinal tissue as surrogate for human tissue, e.g. by scaling using PBPK modelling. (Puccinelli et al., 2010, Kleine et al., 2008, Wiercinska et al., 2012).
To study the intestinal absorption and gut health in a more physiologically relevant model using intestinal tissue, we recently developed an improved alternative for the Ussing chamber system, the InTESTine™ system (Westerhout et al., 2014). Due to rather limited availability of human intestinal tissue, we initially set-up and evaluated the InTESTine™ system with porcine intestinal tissue. The application of human donor intestinal tissue in InTESTine™ was only added recently, and will possibly fasten the translation to the human *in vivo* situation and enables the study of human specific intestinal targets (unpublished data). Data from the current study will further improve our understanding of the observed differences in the intestinal absorption and metabolism of various drugs and nutrients between these different preclinical intestinal models. We revealed some important differences between Caco-2 cells, porcine intestinal tissue and human intestinal tissue that need to be taken into account when using one of these models, for example, by scaling the differential expression of these transporter proteins and metabolizing enzymes to human tissue. We have recently shown the value of absolute transporter protein expression determination for IVIVE (Bosgra et al., 2014), where we predict hepatic disposition of rosuvastatin by scaling from individually transfected cell lines by correcting for absolute transporter protein expression within the plasma membrane. Therefore, as a next step, these data will be integrated into *in silico* models to the use of IVIVE in order to better predict processes that determine intestinal absorption and finally predict oral bioavailability of orally administrated compounds.
ACKNOWLEDGEMENTS

The authors thank A. van Adrichem, B. Blauboer, J. Bogaards, M. Bol-Schoenmakers, A. Gootzen, H. Jansen, I.H.G. Nooijen, F. Schrander, and M. Verwei for their excellent scientific and technical assistance, and Prof. G.M.M. Groothuis (University of Groningen, The Netherlands) for kindly providing the human intestinal samples.
AUTHORSHIP CONTRIBUTIONS

Participated in research design: Vaessen, Lipzig, Pieters, Krul, Wortelboer, van de Steeg

Conducted experiments: Vaessen, van de Steeg

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The work described was funded by the Dutch Government, Ministry of Economic Affairs and the Province and Municipality of Utrecht (PID101063).
FIGURE LEGENDS

Figure 1. Expression of various uptake and efflux transporter proteins within the plasma membrane of different regions of the pig intestinal tract presented as absolute expression (A) and expression relative to villin (B), as well as the relative protein expression in respectively duodenum (C), jejunum (D) and ileum (E) ranked in increasing order according to protein expression levels. Data are presented as mean ± SEM (n=4, samples processed in duplicate). *, p < 0.05; **, p < 0.01; ***, p < 0.001 when compared to expression level in duodenum.

Figure 2. (A) Relative expression of various uptake and efflux transporter proteins within the plasma membrane of human jejunum, pig jejunum and Caco-2 cells (normalized for the amount of epithelial cells using villin as epithelial marker protein). (B) Comparison of absolute expression levels of various uptake and efflux transporter proteins within the plasma membrane of human and pig jejunum. Data are presented as mean ± SEM (tissue samples n=4, samples processed in duplicate, Caco-2 cells n=2, samples singly processed). *, p < 0.05 when compared between human and pig intestinal tissue; #, p<0.05; ###, p<0.001 when compared between Caco-2 cells and human intestinal tissue.

Figure 3. Gene expression of different CYP enzymes in human jejunum, human Caco-2 cells and pig jejunum. Gene copy numbers are corrected for beta-actin (ACTB) and villin (VIL) copy numbers to correct for input material. Data are presented as mean ± SEM (n=9 for human jejunum, n=10 for Caco-2 cells and n=5 for pig jejunum) and samples were processed in duplicate. Na, not applicable (no pig homologue available)

Figure 4. Gene expression of different UGT enzymes in human jejunum, human Caco-2 cells and pig jejunum. Gene copy numbers are corrected for beta-actin (ACTB) and villin (VIL) copy numbers to correct for input material. Data are presented as mean ± SEM (n=9 for human jejunum, n=10 for Caco-2 cells and n=5 for pig jejunum) and samples were processed in duplicate. Na, not applicable (no pig homologue available)
### Table 1. Homology between human CYP enzymes and pig variants

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Table 2. Homology between human UGT enzymes and pig variants

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Figure 1
Figure 2
Figure 3
Figure 4