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TITLE PAGE

In Vitro-In Vivo Extrapolation Scaling Factors for Intestinal P-glycoprotein and Breast Cancer Resistance Protein: Part I: A Cross- Laboratory Comparison of Transporter Protein Abundances and Relative Expression Factors in Human Intestine and Caco-2 Cells

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RUNNING TITLE PAGE

Running Title: IVIVE Scalars for Intestinal Transporters

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ABBREVIATIONS – AQUA (Absolute QUAntification); AUC (Area Under the Concentration-Time Curve); BCRP (Breast Cancer Resistance Protein); BLQ (Below Limit of Quantification); C_{max} (maximal systemic plasma Concentration); E (Glutamic Acid); Glu-Fib (Glu-Fibrinopeptide B); HP (High Passage); IVIVE (In Vitro-In Vivo Extrapolation); K (Lysine); LC-MS/MS (Liquid Chromatography tandem Mass Spectrometry); LLOQ (Lower Limit Of Quantification); LP (Low Passage); MRM (Multiple Reaction Monitoring); Na/K-ATPase (Sodium/Potassium-ATPase); NNOP (Non-Naturally Occurring Peptide); NBD (Nucleotide Binding Domain); OATP (Organic Anion Transporting Polypeptide); P_{app} (apparent Permeability); P-gp (P-glycoprotein); PBPK (Physiologically-Based Pharmacokinetic); QconCAT (Quantification CONcatamer); QTAP (Quantitative Targeted Absolute Proteomic); R (Arginine); REF (Relative Expression Factor); REF_{iBCRP} (Relative Expression Factor for intestinal Breast Cancer Resistance Protein); $REF_{iNa/K\text{-}ATPase}$ (Relative Expression Factor for intestinal Sodium/Potassium-ATPase); $REF_{iP\text{-}gp}$ (Relative Expression Factor for intestinal P-glycoprotein); SILAC (Stable Isotope Labelling of Amino Acids in Culture); SILAM (Stable Isotope Labelling of Amino Acids in Mammals); SNP (Single Nucleotide Polymorphism).

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ABSTRACT

Over the last 5 years the quantification of transporter protein absolute abundances has dramatically increased in parallel to the expanded use of *in vitro – in vivo* extrapolation (IVIVE) and physiologically-based pharmacokinetics (PBPK) linked models, for decision making in pharmaceutical company drug development pipelines and regulatory submissions. Although several research groups have developed laboratory-specific proteomic workflows, it is unclear if the large range of reported variability is founded on true inter-individual variability or experimental variability, due to sample preparation, or the proteomic methodology used. To assess the potential for methodological bias on end-point abundance quantification, two independent laboratories, the University of Manchester (UoM) and Bertin Pharma (BPh), employing different proteomic workflows, quantified the absolute abundances of Na/K-ATPase, P-gp and BCRP in the same set of biological samples from human intestinal and Caco-2 cell membranes. Across all samples, P-gp abundances were significantly correlated ($p = 0.04$, $Rs = 0.72$) with a 2.4-fold higher abundance ($p = 0.001$) generated at the UoM compared to BPh. There was a systematically higher BCRP abundance in Caco-2 cell samples quantified by BPh compared to UoM, but not in human intestinal samples. Consequently, a similar intestinal relative expression factor (REF), based on distal jejunum and Caco-2 monolayer samples, between laboratories was found for P-gp. However, a 2-fold higher intestinal REF was generated by the UoM (2.22) versus BPh (1.11). We demonstrate that differences in absolute protein abundance are evident between laboratories and those are likely to be founded on laboratory-specific methodologies relating to peptide choice.

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INTRODUCTION

Numerous laboratories utilising a diverse range of techniques are quantifying the absolute abundance of proteins by quantitative targeted absolute proteomic (QTAP) strategies in mammalian tissues and *in vitro* cell systems. Up to ten-fold differences in absolute abundances have been observed for specific drug transporter isoforms in independent samples between laboratories, for example, hepatic organic anion transporting polypeptide (OATP) OATP1B1 (Prasad et al., 2014; Vildhede et al., 2014). Differences in the proteomic workflow including techniques required to: (i) select and generate the peptide standard(s); (ii) generate an enriched membrane fraction; (iii) denature and digest the proteins; (iv) separate the peptides by chromatography and (v) conduct mass analysis of the selected peptides and peptide fragments (LC-MS/MS) may underlie this variability.

Studies have investigated the impact of methodological differences on quantification of transporter protein abundances. For example, the type of solubilizing agent used prior to protein digestion has been shown to influence the quantification of OATP proteins in HEK293 transfected cells (Balogh et al., 2013) and can be a significant source of variability between studies. The selection of the peptide standard is also likely to be important and its influence on protein abundance quantification requires verification. In a recent study, the choice of peptide standard was considered to bias end-point abundance quantification of hepatic OATP1B1 (Terasaki et al., 2014). However, in a comparable study by Prasad et al., there was also an impact of peptide choice on OATP1B1 abundance using two of the same peptides as Terasaki et al., 2014, yet little influence of peptide choice for P-glycoprotein (P-gp), Breast Cancer Resistance Protein (BCRP), OATP1B3 and OATP2B1 was observed (Prasad et al., 2014). The digestion strategy employed might also influence the capacity to

quantify protein abundances and to generate peptides that suffer missed-cleavage by trypsin (Chiva et al, 2014). Studies comparing different proteomic approaches within a laboratory found that when comparing immunoblotting to stable isotope labelling of amino acids in culture/mammals (SILAC/SILAM) techniques, there was a reasonable agreement within the same samples to the absolute quantification (AQUA) method routinely employed (Kamiie et al., 2008; Qiu et al., 2013; Prasad and Unadkat, 2014a).

After showing a direct relationship between activity and abundance, the absolute transporter protein abundance and the inter-individual variability are required in order to generate representative populations within Physiologically-Based Pharmacokinetic (PBPK) models. Furthermore, a relative expression factor (REF), the ratio of the transporters' in vivo expression to that in the in vitro system, enables the impact of transporter activity on oral drug absorption to be assessed using an IVIVE-PBPK strategy (Harwood et al., 2013). Establishing if differences in abundances reported across laboratories are derived from inter-individual variability of a sample set or population, variability associated with assay-specific techniques and/or specific data analysis methods within each laboratory are crucial for generating robust system parameters that can be employed in PBPK models that represent *in vivo* abundances. Considering the dramatic increase in the use of IVIVE-PBPK models for decision making in pharmaceutical company drug development pipelines, as well regulatory submissions over the last 5 years (Huang et al., 2013; Shepard et al., 2015), better insight into the comparability of various quantification of transporter protein absolute abundances is becoming essential.

To address if there are laboratory-specific differences in generating system parameters for undertaking transporter-mediated IVIVE-PBPK, a multi-center study evaluating the consistency and comparability of the preparation steps and analytic outcome has been advocated (Harwood et al., 2014). However, to our knowledge, studies comparing the

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quantification of absolute transporter protein abundances and the subsequent generation of REFs between laboratories for the same samples have not been reported in the literature yet. While not within the scope of this article, the impact of those laboratory–specific REF values on predicted pharmacokinetic outcomes in an IVIVE-PBPK model are provided in an accompanying study (Harwood et al., 2015, submitted). The aim of this study was to compare the absolute transporter abundances for 3 transporter proteins; Na/K-ATPase, P-gp and BCRP in matched Caco-2 cell and human intestinal total membrane fractions, quantified by two independent laboratories, the University of Manchester (UoM), Manchester, UK, and Bertin Pharma (BPh), Orleans, France, in which each laboratory utilised different QTAP workflows. These data were used to generate REFs for intestinal Na/K-ATPase ($REF_{iNa/K\text{-}ATPase}$), P-gp ($REF_{iP\text{-}gp}$) and BCRP ($REF_{iBCRP}$) based on the distal jejunal and Caco-2 absolute abundance specific to each laboratory.

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MATERIALS and METHODS

Human Intestinal Tissue

Human intestinal tissue (*n* = 3 distal jejunum and *n* = 1 distal ileum) was obtained after informed consent from patients undergoing elective intestinal surgery at Salford Royal NHS Foundation Trust, Salford, UK. Prior ethics committee approval had been granted by the North West Research Ethics Committee, UK, (12/NW/0306) and all procedures were carried out in accordance with the Declaration of Helsinki guidelines. Patients suffering from inflammatory bowel disease (i.e., Crohn's disease or ulcerative colitis) and/or known to be affected by hepatitis B were excluded from participation. Donor demographics and drug history have are provided in the Supplemental Information (Table S1).

Cell Culture

Caco-2 cells were purchased from the American Type Tissue Culture Collection (ATCC, Rockville, MD, USA) at passage 18. Additionally, cryopreserved higher passage Caco-2 cells were used, that were originally obtained from the ATCC and were subsequently cultured to a passage greater than 100 in the laboratories of the Biomedical Facility, Salford Royal Hospital Trust, Salford, UK. Cell culture reagents and transporter buffers, Dulbecco's Modified Eagle's Medium (DMEM), penicillin & streptomycin, L-glutamine, non-essential amino acids and Hanks Balanced Salt Solution (HBSS) were purchased from Invitrogen Life Sciences (Paisley, Scotland). New-born foetal calf serum gold (FCS) (heat inactivated) and trypsin- ethylenediaminetetraacetic acid (EDTA) were purchased from PAA Laboratories (Yeovil, UK). Low (LP, passage 25-35) and high passage (HP, passage 111) Caco-2 cells

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were maintained in complete DMEM growth media containing 10% FCS, 45 U/mL penicillin, 45 µg/mL streptomycin, 1% non-essential amino acids and 1.1% L-glutamine in an humidified atmosphere of 95% air and 5% CO_2 at 37°C. The cells were fed every 2 days with fresh growth media until 90% confluent where they were sub-cultured routinely every 6 days with trypsin (0.05%)-EDTA and seeded into 75 or 175 cm^2 adherent tissue culture flasks at 12,000 cells per cm^2. Caco-2 cells were seeded onto 44 cm^2 Transwell filters (CC3419, Corning, Lowell, MA, USA) at 220,000 cells per cm^2 (*n*=3 pooled filters per experiment) and cultivated for 21 days (*n*=4 experiments, including a single HP Caco-2 experiment) or 29 days (*n*=3 LP experiments) with feeding every second day with complete DMEM. Caco-2 monolayer integrity was assessed by measurement of trans-epithelial electrical resistance (TEER) and permeability of the paracellular marker lucifer yellow (LY; 50 µM). LY permeability was measured in an apical-to-basolateral transport direction over 60 min at 37°C. Only monolayers showing significant TEER and LY apparent permeability (P_{app}) of ≤ 0.5 x 10^{-6} cm/s were used.

Human Enterocyte and Caco-2 Total Membrane Fractionation

Total membrane preparations were isolated by differential centrifugation after eluting human enterocytes by a calcium chelation protocol (Harwood et al., 2015). Caco-2 cell monolayers total membrane preparations were obtained after overnight lysing by differential centrifugation (Russell et al., 2013). Protein content was determined by the BCA assay.

Shipping/Transfer of Total Membrane Fractions

Total membrane samples were treated differently based on the specific protocols employed within each laboratory.

Total membrane fractions stored at -80°C were either retained at the UoM for subsequent digestion and LC-MS/MS analysis, or shipped by freight overnight on dry ice to BPh. Upon receipt of the consignment of total membrane fractions by BPh, samples were immediately stored at -80°C. The total membrane fractions retained by the UoM were also transferred on ice (approximately 45 min) from Salford Royal Hospital Trust to the Manchester Institute of Biotechnology where, following storage at -80°C, subsequent proteolytic digestion and LC-MS/MS analyses were performed. Analysis of total membrane proteins samples by BPh was performed blind, whereas analysis at the UoM was performed with knowledge of sample type. Final combined analysis of the quantified samples was performed at the UoM.

Proteolytic Digestion

At BPh, total membrane proteins (50 μg) were digested by the MS2 Plex assay kit developed by BPh and based on techniques developed at Tohoku University, Sendai, Japan including reduction, alkylation and an overnight tryptic digestion step (Kamiie et al., 2008, Kunze et al., 2014). The digestion strategy at the UoM was an adapted in-solution digest of 50 μg of total membrane protein based on established methods (Balogh et al., 2013) and also accounts for peptide losses by a gravimetric method (Harwood et al., 2015). The digestion and analysis of the samples took place within a 1 month period between laboratories, a time period considered unlikely to impact on end point abundance determinations (Sakamoto et al., 2011).

Determination of Transporter Abundances

Different QTAP workflows were employed within each laboratory; BPh employ an AQUA approach whereas the UoM utilise a Quantification CONcatamer (QconCAT) approach. The key differences between the AQUA and QconCAT approaches employed by BPh and UoM, respectively, are that (i) AQUA standard peptides are generated by chemical synthesis

whereas QconCAT peptides are metabolically generated and labelled in a biological host vector (i.e., *E. coli*) and (ii) QconCAT constructs possess non-naturally occurring peptide (NNOP) internal standards enabling the concentration of the target labelled standards released in equimolar concentrations upon proteolytic digestion to be quantified, whereas AQUA peptide(s) concentrations are known prior to their entrance into the QTAP workflow and abundances are calculated against a calibration curve. Therefore, in this study the QconCAT construct is simultaneously digested with the total membrane fractions containing the analytes.

An overview of the key components of the QTAP workflows between each laboratory is shown in Table 1. At each stage, a different strategy, protocol, peptide choice or analytical instrument was employed between laboratories as follows:

Bertin Pharma Methods. Transporter protein abundances for Na/K-ATPase, P-gp and BCRP were determined by BPh utilising an AQUA-based simultaneous multiple reaction monitoring (MRM) LC-MS/MS strategy. The SIL proteotypic peptides selected for analysis were AAVPDA[**V**^{13}C,^{15}N]GK (Na/K-ATPase), FYDPL[**A**^{13}C,^{15}N)GK (P-gp) and SSL[**L**^{13}C,^{15}N]DVLAAR (BCRP). The selected ions and transition schedules for these peptides have been published (Kamiie et al., 2008; Sakamoto et al., 2011). The assay mixture (60 μL) prior to analysis consisted of the digested peptides (50 μL) and labelled standards (10 μL). Isotope-labelled standards (10 μL) were added to the peptide mixture to obtain a mixture with a specific relationship after proteolytic digestion. Samples were analysed by LC-MS/MS using a normal flow series 200 autosampler/HPLC pump and a Flexar LC (Perkin Elmer, Waltham, MA, USA) coupled to a API5500 triple quadrupole mass spectrometer (AB Sciex, Framingham, MA, USA). The assay mixture (40 μL) for each sample was injected into the LC system analytical column (X Bridge BEH130 C18, 100 mm x 1.0 mm, 3.5 μm, Waters, UK) with column oven maintained at room temperature. Peptides were eluted with a 50 μL.

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min^{-1} flow rate and a gradient of 2 to 60% acetonitrile over 60 min. The transition schedules were obtained by direct flow injection of peptide solutions. The $[M+2H]^{+2}$ ion was selected as parent ion (Q1) and the 4 most intense fragment ions obtained by collision induced dissociation in Q2 were selected as the Q3 transitions.

University of Manchester Methods. Transporter protein abundances were determined by UoM using a QconCAT-based simultaneous MRM LC-MS/MS strategy (Russell et al., 2013; Harwood et al., 2015).

All protein abundances are given as femtomol of transporter per microgram (fmol/μg) of total membrane protein, where for BPh analysis, the denominator is the nominal protein mass (typically 50 μg) entering the digestion phase of the workflow, and for the UoM it is the peptide content entering the sample tube for injection onto the chromatography column after correction for losses by a gravimetric method (Harwood et al., 2015).

The choice of peptides within the entire protein sequence for Na/K-ATPase, P-gp and BCRP is provided in Figures S1, S2 and S3 in the Supplemental Information.

Statistical Analysis

The abundance data were tested for normality using the Kolmogorov-Smirnov normality test. Due to the negative results of the normality test and the relatively small sample size (n=10-11), only non-parametric statistical tests were used. Transporter absolute abundance data from each laboratory were compared by a Wilcoxon matched pair analysis. Spearman's rank order analysis was used to test for sample abundance correlations. The statistical significance of the correlations was assessed using t-distribution analysis. An α value of 0.05 was employed to indicate a significant difference in all analyses. The objectivity of the study was

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maintained by keeping the proteomic analysts at both BPh and UoM blinded as to the results of the abundance quantifications on the matched samples.

Relative Expression Factor (REF) Generation

Based on abundance data in the distal jejunum samples (*n*=3) and 21d filter-grown Caco-2 monolayers (*n*=3 experiments on different days, based on *n*=3 pooled filter per experiment), a REF for IVIVE was generated (using Equation 1) for all 3 proteins. The REFs were based on 21d, rather than 29d-grown Caco-2 monolayers, as a monolayer of this age is more commonly used for drug transport assays in the industry.

$$REF = \frac{\text{Absolute } In\ Vivo \text{ Expression}}{\text{Absolute } In\ Vitro \text{ Expression}} \qquad \text{Equation 1}$$

Although absolute expression values were generated in this study, they were set relative to each other (Equation 1), thus one REF was obtained for each of the proteins (Na/K-ATPase, P-gp and BCRP) for each laboratory.

RESULTS

Inter-Laboratory Method Differences between Groups Quantifying Transporter Protein Absolute Abundances

To give perspective to this cross-laboratory study, a literature analysis of the diversity of techniques used by different laboratories for transporter absolute abundance quantification was undertaken. Fifteen laboratories have reported quantification of transporter abundances in mammalian tissues or in in vitro systems. Table 2 highlights the general differences in methods for sample preparation, peptide selection, digestion strategies and analytical systems and shows that there is considerable variation in the methodologies used to quantify transporter abundances. The predominant approach has been to use synthetically made isotope labelled standard peptides like AQUA. However, a combination of label free and targeted (labelled) approaches have also been utilized simultaneously within the same study (Karlgren et al., 2012). BPh provides QTAP services and kits based on technologies developed at Tohoku University and, as a result, the workflows from both laboratories share many similarities. In fact, several laboratories foster links with the forerunning developers of these methodologies, i.e., Tohoku University and Pfizer Ltd., (Groton, CT, USA). Peptides that are used as surrogates for the quantification of the complete protein are selected based on a variety of criteria (Ohtsuki et al., 2011; Oswald et al., 2013; Prasad and Unadkat, 2014b; Qiu et al., 2014). Consequently, for the frequently quantified transporter protein P-gp, three peptides are routinely employed to quantify human P-gp (AGAVAEEVLAAIR/ NTTGALTTR/ FYDPLAGK; Table 2) highlighting a current lack of consensus among groups. Comparative studies testing all three peptides on matched samples between laboratories have yet to be reported. In-solution trypsin-based overnight digestion strategies dominate, but the utility of Lys-C to facilitate protein digestion prior to incubation with trypsin is favored by The Max Planck Institute (Martinsried, Germany) and the UoM (Achour

and Barber, 2013). Nano and normal flow LC approaches are used but the advantages of one approach over another have not been discussed within these studies. The triple quadrupole MS system is routinely employed for targeted approaches, however the manufacturers and models differ between laboratories. For comparative purposes, the use of quantitative immunoblotting is also incorporated in this analysis. This approach does not employ peptide selection strategies, protein digestion and LC-MS/MS for analysis, but relies on selective antibodies being available for target transporters, which is not always the case (Tucker et al., 2012).

Cross Laboratory Comparison of Transporter Absolute Protein Abundances

Quality Control: The analytical quality control for Na/K-ATPase, P-gp and BCRP was established for linearity ($R^2 \geq 0.999$), precision (CV≤15%), and accuracy (relative error, 71-112% using quality control samples) and the lower limits of quantification (LLOQ) was determined as 0.125 fmol/μg for all proteins by BPh (see Tables S2-S4 in the Supplemental Information). The linearity and LLOQ were based on synthetic labelled standard peptides with the precision determined by the coefficient of variation across the 4 selected transitions of each standard peptide quantified.

The analytical quality control for the same proteins established at the UoM has been reported in detail by Harwood and co-workers (Harwood et al., 2015). The linearity for abundance determination of each peptide in the presence of a biological matrix (i.e., human intestinal total membrane protein) was established. In addition, linearity in the quantification of the QconCAT used by UoM was determined using the internal calibrator NNOP peptide Glu-Fibrinopeptide B (glu-fib) (Harwood et al., 2015). The intra-day and inter-day precision and relative error across biological samples was also measured as part of the quality control procedure. The LLOQ for abundance determinations at UoM was 0.2 fmol/μg for all proteins.

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Appraisal of Na/K-ATPase, P-gp and BCRP Peptides Selected by BPh and UoM: The proteotypic standard peptides selected by each laboratory for quantification of Na/K-ATPase, P-gp and BCRP were based on criteria published by Tohoku University and the UoM (Kamiie et al., 2008; Russell et al., 2013). For Na/K ATPase (ATP1A1), the peptide selection by BPh (AAVPDAVGK) was not favorable for selection by UoM because of the potential for peptide mis-cleavage as flagged the CONSeQuence program developed at the UoM (Lawless and Hubbard, 2012). Concerns were also raised regarding the peptides uniqueness for quantification purposes, as AAVPDAVGK also occurs in human Na/K-ATPase α2 subunit (Shull and Lingrel., 1987), a protein expressed at low levels in human liver tissue (http://www.proteinatlas.org/ENSG00000018625-ATP1A2/tissue). The position of the selected standard peptides within P-gp and BCRP protein structure is shown in Figure 1. The criteria defined by Tohoku University would have rejected the P-gp peptide selected by UoM as a single nucleotide polymorphism (SNP) is present at position 261 (p.I261V – c.A781G). This SNP has an allelic frequency in African Americans of 0.6% (Kroetz et al., 2003) and 6.9% in Ugandans (Mukonzo et al., 2010), but is not detected in other populations including Caucasians (n = 100), Asian Americans (n = 30), Mexican Americans (n = 10), Pacific Islanders (n = 7) and Japanese (n = 145) (Kroetz et al., 2003; Ozawa et al., 2004). The standard peptide selected for P-gp quantification by BPh was flagged as having the potential for a trypsin missed-cleavage event by CONSeQuence (Lawless and Hubbard, 2012). In the event of missed cleavage, the selected peptide would be elongated, changing its mass, resulting in the first mass filter (Q1) of a triple quadrupole MS, rejecting its selection for downstream collision fragmentation (Q2) and product ion monitoring (Q3). As a consequence, a lower native peptide signal compared to the standard peptide might be expected, in turn generating a lower biological abundance.

The BCRP peptides selected by both groups showed no evidence of SNP's. The peptide selection criteria applied by Tohoku University are unlikely to have scored the UoM peptide highly due to the presence of glutamine (Q) which has the potential to suffer a deamidation post-translational modification. This event was judged tolerable at UoM, given that the rate of glutamine deamidation is considerably lower than that for asparagine (N), with a reaction half-life of 660 days (Li et al., 2010b). The selection criteria employed by UoM would have rejected the BPh BCRP peptide standard due to the anticipated difficulty of efficient trypsin cleavage at dibasic and tribasic tryptic sites, i.e., sequential lysine or arginine residues or lysine and arginine side-by-side at the N-terminal region of the BPh peptide (Lawless and Hubbard, 2012).

Transporter Abundances in Caco-2 and Human Intestinal Samples: Total membrane fractions from seven Caco-2 cell monolayers, three human distal jejunum and a single human distal ileum were analysed in each laboratory (n = 11). The comparative absolute abundances and the correlations between BPh and UoM for Na/K-ATPase, P-gp and BCRP are provided in Figures 2 and 3, respectively. The individual abundance data for each sample is provided in Table 3. For Na/K-ATPase and BCRP, there was no difference in the mean abundances (p = 0.36 and 0.76, respectively, Wilcoxon matched pair test) with a moderate correlation for Na/K-ATPase (Rs = 0.42) and no correlation for BCRP (Rs = 0.39) using a Spearman Rank correlation analysis across all samples between laboratories. A systematically higher BCRP abundance was quantified by BPh compared to UoM in 21 and 29d cultivated Caco-2 cells ranging from 20 to 129% in individual samples (Table 3). This was not reciprocated in the intestinal BCRP abundances, which were on average 17% higher (range; -40 to 72%) in the UoM analysis. A systematically and significantly higher (63%, p = 0.001, Wilcoxon matched pair test) P-gp abundance was quantified by UoM compared to BPh across all samples and, in contrast to Na/K-ATPase and BCRP, P-gp abundances were highly correlated (Rs = 0.72, p =

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0.04, Figure 3B). For the single distal ileum sample, P-gp abundance was below the LLOQ (< 0.125 fmol/µg) in BPh determinations, but the UoM determined a value of 0.2 fmol/µg total membrane protein. In both laboratories, lower mean abundances of P-gp were observed in the human intestinal samples than Caco-2 cell monolayers, irrespective of cultivation time. This is in contrast to BCRP, in which the opposite relationship was observed. The importance of correcting for losses of peptides during reduction, alkylation and digestion steps when determining absolute transporter abundances has been established (Harwood et al., 2015). These corrections were applied only to abundance determinations at the UoM, since they are not a routine component of abundance determinations at BPh. When peptide loss corrections were not applied to samples quantified at the UoM, P-gp abundances were still systematically and significantly higher (52%, p = 0.001, Wilcoxon matched pair test), and Na/K-ATPase and BCRP abundances were also still not significantly different (p = 0.36 and p = 0.58, respectively, Wilcoxon matched pair test).

Generation of Relative Expression Factors (REF): The potential impact of these results on IVIVE scaling was investigated by generating a REF (Equation 1) for human distal jejunum samples and 21d cultivated Caco-2 cell monolayers (*n* = 3, Table 3). Due to the high correlation observed between laboratories when quantifying P-gp abundances, the systematically higher P-gp levels measured by the UoM had no effect on the REF between laboratories (BPh-$REF_{iP\text{-}gp}$ = 0.37, UoM-$REF_{iP\text{-}gp}$ = 0.4). Na/K-ATPase, although exhibiting a moderate correlation (Rs = 0.42), also showed little difference in jejunal-REF between laboratories (BPh-$REF_{iNa/K\text{-}ATPase}$ = 0.68, UoM-$REF_{iNa/K\text{-}ATPase}$ = 0.58). However, BCRP showed distinct differences with the UoM-REF_{iBCRP} being 2-fold higher than the BPh-REF_{iBCRP} (REF were 2.22 *vs*. 1.11, respectively) which is likely to result from the systematically lower Caco-2 cell abundance levels measured at UoM.

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DISCUSSION

Several laboratories have developed and validated protocols for quantifying transporter protein abundances in biological systems using proteomic approaches (Table 2). With this raft of abundance data generated across multiple laboratories now being incorporated into IVIVE-PBPK strategies (Bosgra et al., 2014; Kunze et al., 2014; Prasad et al., 2014; Vildhede et al., 2014), it is critical to establish if laboratory-specific methodologies contribute to a bias in abundance determination. To address this, membrane protein samples from human intestine and Caco-2 monolayers were analysed independently by two laboratories, BPh and UoM. For P-gp, there is a systematic bias in abundances between laboratories. For Na/K-ATPase and BCRP, mean abundances reported by BPh and UoM were similar across all samples; however the limited correlation in abundances of these transporters in individual samples suggests a potential influence of technical and/or analytical variability in post-membrane processing between laboratories.

Efficient protein digestion is critical for abundance determination and integral membrane transporter proteins are notoriously challenging to digest due to their poor solubility (Mirza et al., 2007). Therefore, different solubilizing agents are often used to enhance protein solubility facilitating target protein digestion. Based on the findings of Balogh et al. (2013), UoM employed sodium deoxycholate as the solubilizing agent. However, BPh, do not disclose the components of their MS2Plex digestion kit, and therefore, a systematic evaluation of the effect of potential differences in the procedures employed in both laboratories to denature, reduce, alkylate and digest the proteins cannot be undertaken.

Critical to the quantification of transporter protein abundances is that enzymatic digestion is complete (Ji et al., 2012). The digestion efficiency for selected peptides has been assessed in several studies (Kamiie et al., 2008; Li et al., 2008; Zhang et al., 2011; Ji et al., 2012; Balogh

et al., 2013; Gröer et al., 2013; van de Steeg et al., 2013). However, assessing digestion completeness is a significant challenge due to the limited availability of purified transporter proteins used to monitor peptide digestion (Prasad et al., 2014; Harwood et al., 2015). Peptide instability over the duration of digestion should be considered and could be a factor in MS signal reduction (van den Broek et al., 2013). The digestion strategy at UoM incorporated a lysyl-endoproteinase as a priming step prior to trypsin incubation, cleaving the C-terminal side of lysine residues, generating lysine terminated peptides to improve trypsin digestion efficiency (Achour and Barber, 2013). Within this study, it is assumed, based on prior optimization, that digestion achieves a steady-state over the time course of incubation with the proteolytic enzymes within the biological matrices under investigation for both laboratories; therefore, any source of variability between samples should not result from inefficient digestion.

Selecting peptides that uniquely identify the targeted protein based on trypsin digestion are required. The peptide (AAVPDAVGK) selected by BPh to quantify Na/K-ATPase (ATP1A1) is not unique as the sequence is also found in human Na/K-ATPase α2 subunit (ATP1A2) (Shull and Lingrel., 1987). Peptides possessing a lower potential for missed cleavage events are also advantageous (Lawless and Hubbard, 2012). This is pertinent given that in relative quantification analysis, up to 46% of peptides generated mis-cleaved events after in-solution trypsin digestion in *E. coli* (Chiva et al., 2014). For the peptide (AAVPDAVGK) selected for Na/K-ATPase quantification by BPh, the C-terminal lysine is closely followed by an arginine (K.CR.), a potential cause of mis-cleavage (Lawless and Hubbard., 2012). Furthermore, peptides in which N-terminal flanking glutamic acid (E) is located 2 positions from trypsin cleavage sites (arginine-lysine (R-K)), are prone to missed cleavage. As a result, the P-gp peptide selected at Tohoku University did not score as favorably as other P-gp peptides selected and used by UoM (Russell et al., 2013). Peptide selection through the triple

quadrupole mass spectrometer is based on pre-defined masses, where any violation of these masses, i.e., by missed cleavage of target peptide, leads to the peptides exclusion in mass filter 1 (Q1), resulting in lower abundance measurements. These events may explain the differences in P-gp abundance quantification between laboratories shown in this study. Interestingly, Miliotis and co-workers (2011) used the same peptide selected by UoM (AGAVAEEVLAAIR) to assess the abundance of P-gp in 29d-grown Caco-2 monolayers, reporting an abundance of 7.89 fmol/µg total membrane protein, similar to UoM (6.92 fmol/µg total membrane protein) and much higher than quantification by BPh (1.97 fmol/µg total membrane protein). This lends support to the conjecture that peptide selection may contribute to bias in abundance measurements.

These observations reinforce the critical importance of peptide choice in abundance determination as demonstrated in studies of hepatic OATP1B1 abundance (Terasaki et al., 2014). A previous study from that group used a peptide located in the ninth transmembrane domain of OATP1B1, a region associated with lower tryptic digestion efficiency (Kamiie et al., 2008), and reported OATP1B1 abundances below detection limits in 8 of 17 human liver samples (Ohtsuki et al., 2012). This was unexpected given that OATP1B1 exhibits the highest abundance of all liver OATP transporters as shown in a recent meta-analysis (Badée et al., 2015). Re-evaluation of the original peptide against 4 other peptides located in intracellular or extracellular loop domains of OATP1B1 showed that this peptide underestimates mean abundance by more than 20-fold in comparison to the best performing peptide (Terasaki et al., 2014). The systematic difference in P-gp abundances quantified by UoM and BPh in this study is smaller (63%, Table 3), however, this level of methodological bias in abundance determination is of concern when incorporating population abundances into PBPK models, given the potential effect on predicted transporter-mediated drug disposition (Harwood et al., 2013).

The consistently lower BCRP abundance in Caco-2 cells reported by UoM in this study (Table 3, Figures 2C and 3C) does not appear to be peptide-specific since BCRP abundances in 3 out of 4 human samples were higher at UoM than BPh. One possible explanation is that the Caco-2 cell membrane matrix may affect digestion of the selected BCRP peptide (Figure 1) impacting on abundance quantification. Chromatographic effects may also contribute to these differences, as reported for NTTGALTTR, a peptide commonly used to quantify P-gp (Gröer et al., 2013). Chromatographic interference is unlikely to be the source of the observed lower BCRP abundances in Caco-2 cells as co-elution profiles (i.e., identical retention times) of native and standard peptides were generated. However, the methodological validation of these peptides was initially performed in human intestine (Harwood et al., 2015). Further studies assessing the optimal digestion conditions for the selected BCRP peptides in Caco-2 cells are warranted. Additionally, this study does not control for different membrane fractionation techniques, which was recently suggested as a source of variability between and within studies (Heikkinen et al., 2015). To test this, repeated membrane extraction, protein digestion and subsequent abundance quantification is required on the same tissue over multiple days. This would remove variability associated with technical/analytical procedures from the intrinsic biological variability, a requirement for building representative IVIVE-PBPK frameworks with population variability.

In addition to obtaining transporter abundances in tissues of individuals for incorporation into PBPK models, abundance data from mammalian tissues and *in vitro* systems is used to generate IVIVE scaling factors (Li et al., 2010a; Karlgren et al., 2012; Vildhede et al., 2014). This study generated laboratory-specific REF-IVIVE scaling factors for all three transporters using abundance data for jejunum and Caco-2 monolayers. This enabled the assessment of laboratory-specific biases in abundance measurements on REF. $REF_{iP\text{-}gp}$ was the same between laboratories due to the consistent bias in P-gp abundances for *in vivo* and *in vitro*

systems. However, if bias in abundance quantification occurs selectively within a biological system between laboratories (i.e., Caco-2 cell BCRP abundances being nearly 2-fold higher at UoM), the laboratory-specific REF is affected and can be significantly different.

The results of this study for the first time show significant differences in transporter protein abundance reported on the same samples by different laboratories, implying methodological bias is likely to be a significant source of variability. Thus, caution should be exercised when utilising absolute mammalian tissue abundances from literature sources to estimate whole organ abundances and REFs in IVIVE-PBPK strategies. Across-laboratory protein abundance comparisons on the same samples can reveal biases that are integral to a particular laboratory, or a laboratory-specific step within a workflow. We recommend further comparison studies using matching samples across a wider range of laboratories, including the complete workflow-specific to each laboratory to establish the critical factors influencing protein abundance quantification. Subsequently, by selecting the technique/operating procedure with the most favorable precision, accuracy and reproducibility, a further study using the same biological samples as the first comparative analysis can commence. The overall aim is to reduce method-dependent variability to an aceptable minimum, so the intrinsic biological variability can be determined with high accuracy in any laboratory using the same workflow.

Finally, to what extent do variations in REF caused by differences in transporter abundance data between laboratories influence the prediction of oral drug absorption by IVIVE-PBPK models? Assessing the impact of REFs from different sources on PK parameters by IVIVE-PBPK strategies is addressed in the accompanying study (Harwood et al., 2015 submitted).

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Authorship Contributions

Participated in research design: Harwood, Achour, Neuhoff, Russell, Warhurst, Rostami-Hodjegan

Conducted experiments: Harwood, Achour

Contributed new reagents & analytical tools: Russell, Carlson

Performed data analysis: Harwood, Achour

Wrote or contributed to the writing of the manuscript: Harwood, Achour, Carlson, Neuhoff, Russell, Warhurst, Rostami-Hodjegan

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FOOTNOTES

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FIGURE LEGENDS

Figure 1: The location and nomenclature of the selected peptides for quantifying P-gp (A) and BCRP (B) absolute protein abundances**.** Bertin Pharma (BPh) and the University of Manchester (UoM) selected peptide sequences (bold residues) are shown in the boxes. The amino acid residues immediately flanking the selected peptides are in plain text. The UoM selected peptide contains the potential to harbor a single nucleotide polymorphism p.I261V (c.A781G) for P-gp which is given as gray and underlined text. The variant 'V' peptide is also provided. The nucleotide binding region (NBD) and N-glycosylated sites are shown in the first extracellular loop of P-gp and the third extracellular loop of BCRP.

Figure 2: Absolute protein abundances of Na/K-ATPase (A), P-gp (B) and BCRP (C) determined by Bertin Pharma (BPh, black) and the University of Manchester (UoM, white) in Caco-2 cell monolayers (n=7) and human intestinal total membrane protein fractions (n=4). The text above the bars indicates Caco-2 monolayer cultivation age. HP is high passage Caco-2 cells. Key: Distal Jejunum (DJ); Distal Ileum (DI); Below the Limit of Quantification (BLQ). * $p < 0.05$. The mean ± standard deviation is given for the DJ, 21d, 29d Caco-2 cells and overall means are given across all samples for each protein.

Figure 3: Correlation analysis (Spearman's rank order test) of the absolute protein abundances of Na/K-ATPase (A), P-gp (B) and BCRP (C) between Bertin Pharma and the University of Manchester. Diamonds denote human (white) and Caco-2 cell monolayers (black). Spearman's rank correlation coefficients (Rs) are provided as text on the plot, as are the p values representing the *t*-distribution for assessing the significance of the correlation. The dashed line is the line of identity and the solid line is the line of best fit. A single sample (distal ileum) from BPh for P-gp was below the limit of quantification.

TABLES

Table 1. An outline of the methods constituting the QTAP workflow specific to each laboratory for the absolute quantification of Na/K-ATPase, P-gp and BCRP

Criteria	The University of Manchester	Bertin Pharma
Peptide Selection	*In Silico* – CONSeQuence program[a]	*In Silico* – Tohoku University algorithm[b]
Standard Generation	Quantitative Concatenation (QconCAT)	Absolute Quantification (AQUA)
Selected Peptides	Na/K-ATPase - IVEIPFNSTN[**K**^{13}C] P-gp - AGAVAEEVLAAI[**R**^{13}C] BCRP - VIQELGLD[**K**^{13}C]	Na/K-ATPase - AAVPDA[**V**^{13}C,^{15}N]GK P-gp - FYDPL[**A**^{13}C,^{15}N]GK BCRP - SSL[**L**^{13}C,^{15}N]DVLAAR
Digestion	Deoxycholate denaturation, Lys-C plus trypsin-based digestion	MS2Plex-based process including trypsin-based digestion
LC-MS/MS	Nano flow LC – nanoAcquity (Waters) with TSQ Vantage (Thermo), MRM	Normal flow LC – Flexar LC (Perkin Elmer) with API5500 (AB SCIEX) Vantage (Thermo), MRM

[a]details of CONSeQuence given in (Eyers et al., 2011) [b]details of peptide selection criteria given in (Kamiie et al., 2008).

Table 2. A comparative analysis of methods used for quantification of transporter protein absolute abundances across research groups

Group	Peptide Selection Method	Standard Generation (P-gp Standard)	Tissue/Cell Fractionation (DC/KIT/FASP)	Detergents/ Chaotropic/ reducing agent	Digestion strategy	Standard Pre/Post Digestion	LC-MS/MS system	Source
Tohoku University (Study with *Boehringer Ingelheim, Japan included)	*In silico* – In-house & MS/MS verification	AQUA Isotope Label (FYDPLAGK)	DC - PM fraction; whole brain capillaries	Guanidinium hydrochloride	In-solution Trypsin (16h, 37°C) E:S-1:100	Post digestion	Multiplex HPLC – normal & nano flow MS - API5000 /QTRAP5500 (AB SCIEX) or 4000 Q trap (Applied Biosystems)	(Ohtsuki et al., 2012) (Sakamoto et al., 2011)* (Uchida et al., 2014)
Pfizer	Prospector – UCSF & MS/MS verification	AQUA Isotope Label (NTTGALTTR)	Kit extraction – native membrane (Calbiochem)	DTT; DOC	In-solution Trypsin (16h, 37°C) E:S-1:20-50	Pre-digestion or post-digestion	Single & multiplex HPLC – normal flow MS - API4000 (Applied Biosystems) †	(Li et al., 2009) (Zhang et al., 2011) (Balogh et al., 2013) (Qiu et al., 2013)
University of Paris Descartes	Linked to Tohoku University	AQUA Isotope Label (NTTGALTTR)	Whole brain capillaries	Guanidinium hydrochloride	In-solution Trypsin (16h, 37°C) E:S-1:100	Post-digestion	HPLC – normal & nano flow MS – 4000 QTRAP/API 5000 (Applied Biosystems)	(Shawahna et al., 2011)
AstraZeneca	MS fragmentation – MASCOT search & MS/MS verification	AQUA Isotope Label (AGAVAEEVLAAIR)	DC – TM fraction	PPS	In-solution Trypsin (6h, 37°C) E:S-1:20	Pre-digestion	Single UHPLC – normal flow MS – 6460 (Agilent Technologies)	(Miliotis et al., 2011)
TNO	See Tohoku University entry	AQUA Isotope Label	DC - PM fraction	DTT	In-solution Trypsin (o/n, +2h, 37°C) E:S-1:100	Post-digestion	Multiplex UHPLC – normal flow MS – Xevo-TQ-S (Waters)	(van de Steeg et al., 2013)
The University of Washington	Prospector – UCSF & Skyline & MS/MS verification Links to Pfizer's proteomic laboratory	AQUA Isotope Label (NTTGALTTR)	Kit extraction – native membrane (Calbiochem)	DTT	In-solution Trypsin (14-24h - 37°C) E:S-1:25	Pre-digestion (Deo et al., 2012) or post digestion	Multiplex UHPLC – normal flow MS – See Pfizer† or 6460A (Agilent Technologies)	(Deo et al., 2012) † (Prasad et al., 2014)
The University of Uppsala	Links to Pfizer & Max Planck Institute proteomic laboratories §	AQUA Isotope Label P-gp quantification not reported to date	Kit extraction – native membrane (Calbiochem)	DOC	In-solution Trypsin (16h, 37°C) E:S-1:20	Post-digestion	See Pfizer entry.	(Karlgren et al., 2012) (Vildhede et al., 2014)

Max Plank Institute	Total protein non-targeted	DDA Label free	DC – TM fraction	SDS/Urea	In-solution FASP Lys-C (o/n) room temp Trypsin – (2h - 37°C)	n/a	Total protein array HPLC – nano flow Orbitrap LTQ (Thermo Scientific)	(Karlgren et al., 2012) (Wisniewski et al., 2014)
The University of Greifswald[a]	Linked to Tohoku University	P-gp quantification not reported to date	DC - PM fraction	Not stated	In-solution Trypsin (16h, 37°C) E:S-1:100	Post-digestion	Multiplex HPLC – normal & nano flow MS - QTRAP5500 (AB SCIEX) or 4000 Q trap (Applied Biosystems)	(Niessen et al., 2010)
The University of Greifswald[b]	Expasy program & MS/MS verification	AQUA Isotope Label (AGAVAEEVLAAIR)	Kit extraction – native membrane (Calbiochem)	DTT	In-solution Trypsin (16h, 37°C) E:S-1:40	Post-digestion	Multiplex HPLC – normal flow MS - API4000 (Applied Biosystems)	(Groer et al., 2013)
Bertin Pharma	Linked to Tohoku University	AQUA Isotope Label (FYDPLAGK, see Sakamoto et al., 2011)	DC – PM fraction	Unknown[‡]	In-solution[‡] Trypsin	Post-digestion	Multiplex HPLC – normal flow MS - API5000 (AB SCIEX)	(Kunze et al., 2014)
The University of Manchester	SRM Atlas CONSeQuence program – UoM & MS/MS verification	QconCAT Isotope Label (AGAVAEEVLAAIR)	DC – PM fraction	DOC-DTT	In-solution Lys-C (4h - 30°C) Trypsin – (18h - 37°C) E:S-1:20	Pre-digestion	Multiplex HPLC – nano flow MS – TSQ Vantage (Thermo Scientific)	(Russell et al., 2013)
University of Kansas/ Stanford University & Eli Lilly	*In silico* – In-house & MS/MS verification	AQUA Isotope Label (NTTGALTTR)	Kit extraction – native membrane (Millipore)	DOC-DTT	In-solution Trypsin (18h, 37°C) E:S-1:20	Post-digestion	Multiplex UPLC – normal flow MS – Xevo TQ-S (Waters)	(Peng et al., 2015)
Bristol Myers Squibb		AQUA Isotope Label	Kit extraction – native membrane (Calbiochem)	DOC-DTT	In-solution Trypsin (16h, 37°C) E:S-1:20	Post-digestion	Multiplex unknown UPLC – normal flow MS – API6500 (AB SCIEX)	(Shen et al., 2015)
The University of Dundee**	n/a	S-tag – Antibody Label free	DC – TM fraction	n/a	n/a	n/a	n/a	(Tucker et al., 2012)

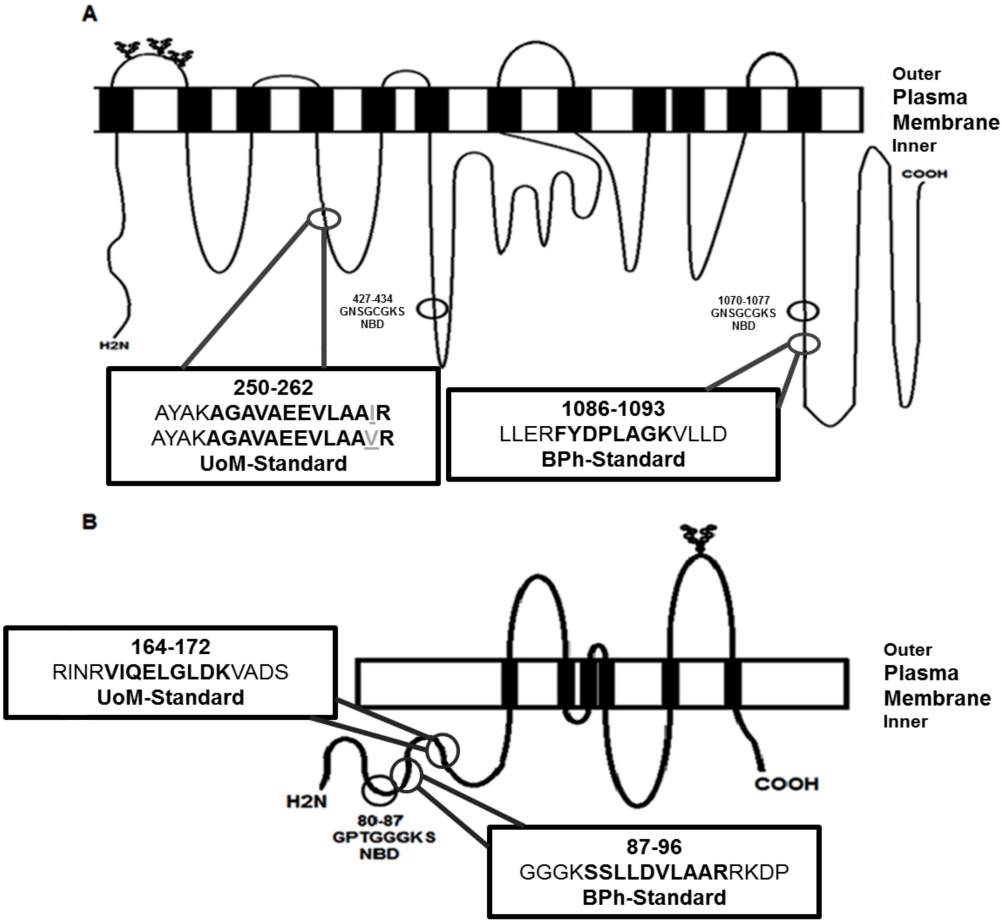
*Boehringer Ingelheim, Japan collaborated with Tohoku University. ** Professor Coughtrie's group are now based at The University of British Columbia. [†]Deo et al., 2012, performed LC-MS/MS analysis at Pfizer Ltd. [‡] An 'off the shelf' MS2Plex kit is used. [§]The targeted proteomic strategy at the University of Uppsala used the Pfizer laboratory and techniques described in Balogh et al., 2013. [a], [b] two distinct groups at The University of Greifswald. Differential Centrifugation (DC); Sodium Deoxycholate (DOC); Sodium Deoxycholate-Dithiothreitol (DOC-DTT); Dithiothreitol (DTT); Filter Aided Sample Preparation (FASP); Plasma Membrane (PM); Total Membrane (TM), Data-Dependent Acquisition (DDA). E:S is the enzyme to substrate ratio for trypsin. PPS is a silent surfactant. The University of California, San Francisco (UCSF), n/a – not applicable, o/n - overnight.

Table 3. Individual abundances of human intestinal and Caco-2 cell monolayer, with REFs for Na/K-ATPase, P-gp and BCRP

	Na/K-ATPase Abundance (fmol transporter/µg total membrane protein)			P-gp Abundance (fmol transporter/µg total membrane protein)			BCRP Abundance (fmol transporter/µg total membrane protein)		
Sample	Bertin Pharma	University of Manchester	Relative Error (%)	Bertin Pharma	University of Manchester	Relative Error (%)	Bertin Pharma	University of Manchester	Relative Error (%)
Distal Jejunum 1	43.39	41.93	-3	1.10	3.07	64	3.19	2.28	-29
Distal Jejunum 2	37.48	34.58	-8	0.40	0.98	59	0.98	1.92	49
Distal Jejunum 3	37.11	29.58	-25	0.80	1.62	51	2.01	3.49	42
Distal Ileum	50.72	24.47	-107	BLQ	0.20	-	0.44	1.6	73
Caco-2 21d (# 1)	51.56	82.47	37	1.89	5.21	64	1.7	1.19	-43
Caco-2 21d (# 2)	56.75	47.16	-20	2.27	4.5	50	1.94	1.16	-67
Caco-2 21d (# 3)	65.86	56.75	-16	2.07	4.31	52	1.94	1.12	-73
Caco-2 29d (# 1)	49.3	77.43	36	1.64	8.27	80	1.34	1.12	-20
Caco-2 29d (# 2)	74.81	54.32	-38	2.29	6.78	66	1.64	0.72	-128
Caco-2 29d (# 3)	52.45	58.83	11	1.98	6.78	71	1.51	0.75	-101
Caco-2 21d HP	90.43	52.33	-73	7.84	5.71	37	2.73	1.64	-66
Mean Distal Jejunum	**39.3 (±3.5)**	**35.4 (±6.2)**	**-12***	**0.8 (±0.3)**	**1.9 (±1.1)**	**58***	**2.1 (±1.1)**	**2.6 (±0.8)**	**17***
Mean Caco-2 21d	**58.1 (±7.2)**	**62.1 (±18.3)**	**-0.4***	**2.1 (±0.2)**	**4.7 (±0.5)**	**55***	**1.9 (±0.1)**	**1.2 (±0.0)**	**-62***
Mean Caco-2 29d	**58.9 (±14)**	**63.5 (±12)**	**3 ***	**2.0 (±0.3)**	**6.9 (±1.3)**	**71***	**1.3 (±0.3)**	**0.9 (±0.2)**	**-83***
Overall Mean	**55.4 (±16.1)**	**50.9 (±18.1)**	**-19***	**2.0 (±2.1)**	**4.8 (±3.5)**	**63***	**1.8 (±0.8)**	**1.5 (±0.9)**	**-33***
REF (Jejunum: Caco-2-21d)	**0.68**	**0.58**	**-**	**0.37**	**0.4**	**-**	**1.11**	**2.22**	**-**

Standard deviations are given in parentheses. * denotes mean relative error when accounting for individual samples. HP, High Passage. BLQ, below limit of quantification.

Figure 1


A
Outer
Plasma
Membrane
Inner
COOH
H2N
427-434
GNSGCGKS
NBD
1070-1077
GNSGCGKS
NBD
250-262
AYAKAGAVAEEVLAAIR
AYAKAGAVAEEVLAAVR
UoM-Standard
1086-1093
LLERFYDPLAGKVLLD
BPh-Standard
B
164-172
RINRVIQELGLDKVADS
UoM-Standard
Outer
Plasma
Membrane
Inner
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H2N
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GPTGGGKS
NBD
87-96
GGGKSSLLDVLAARRKDP
BPh-Standard

Figure 2

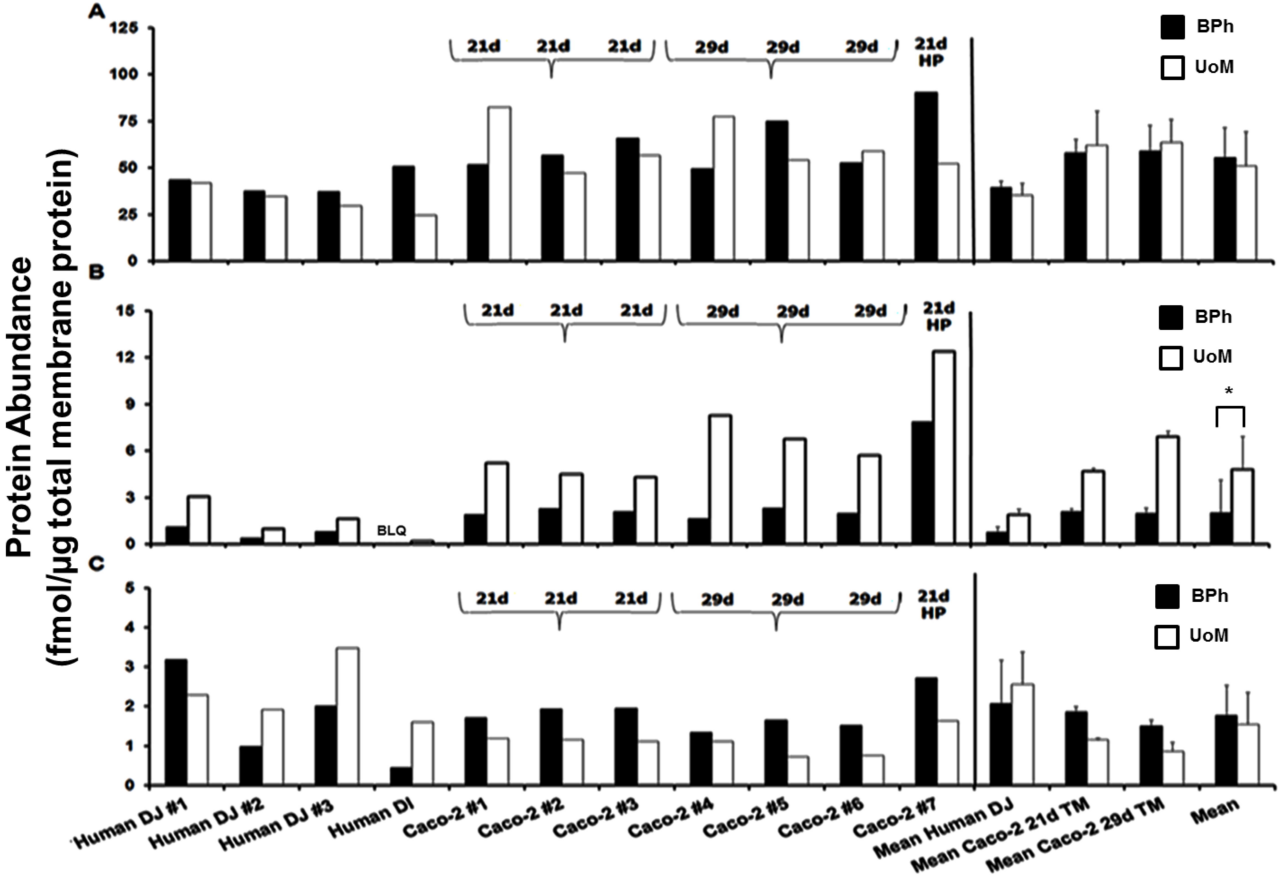
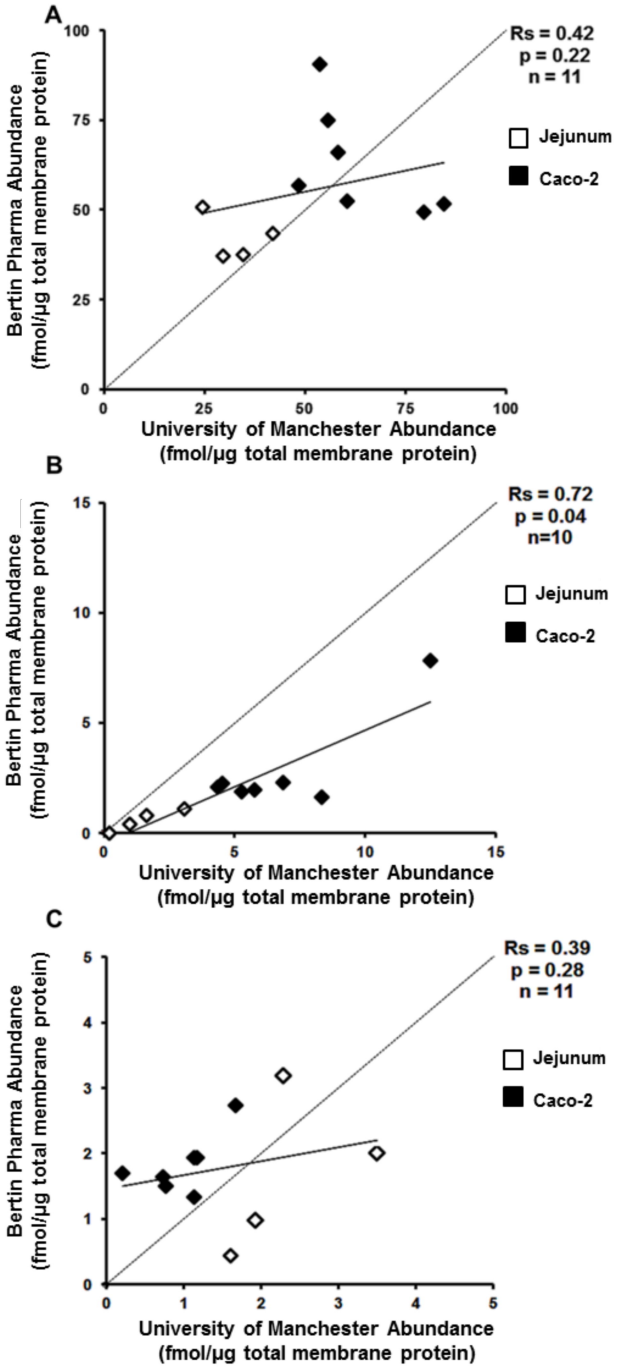

A
B
C
Protein Abundance
(fmol/µg total membrane protein)
125
100
75
50
25
0
15
12
9
6
3
0
5
4
3
2
1
0
21d
21d
21d
29d
29d
29d
21d HP
BPh
UoM
BLQ
*
Human DJ #1
Human DJ #2
Human DJ #3
Human DI
Caco-2 #1
Caco-2 #2
Caco-2 #3
Caco-2 #4
Caco-2 #5
Caco-2 #6
Caco-2 #7
Mean Human DJ
Mean Caco-2 21d TM
Mean Caco-2 29d TM
Mean

Figure 3


A
Rs = 0.42
p = 0.22
n = 11
Jejunum
Caco-2
Bertin Pharma Abundance
(fmol/µg total membrane protein)
University of Manchester Abundance
(fmol/µg total membrane protein)
B
Rs = 0.72
p = 0.04
n=10
Jejunum
Caco-2
Bertin Pharma Abundance
(fmol/µg total membrane protein)
University of Manchester Abundance
(fmol/µg total membrane protein)
C
Rs = 0.39
p = 0.28
n = 11
Jejunum
Caco-2
Bertin Pharma Abundance
(fmol/µg total membrane protein)
University of Manchester Abundance
(fmol/µg total membrane protein)

In Vitro-In Vivo Extrapolation Scaling Factors for Intestinal P-glycoprotein and Breast Cancer Resistance Protein: Part I: A Cross-Laboratory Comparison of Transporter Protein Abundances and Relative Expression Factors in Human Intestine and Caco-2 Cells.
Matthew D Harwood, Brahim Achour, Sibylle Neuhoff, Matthew R Russell, Gordon Carlson, Geoffrey Warhurst, Amin Rostami-Hodjegan.
Drug Metabolism and Disposition

SUPPLEMENTAL INFORMATION

Table S1. Intestinal donor demographic information.

Intestinal Region	Gender	Age	Ethnicity	Smoking Status	Disease/Complication/ Operative Procedure
Distal Jejunum (#1)	Male	65	Caucasian	Unknown	Incisional hernia & fistula
Distal Jejunum (#2) (> 130cm proximal the ileo-cecal valve)	Female	41	Caucasian	Non-smoker	Laparotomy, small bowel resection for enterocutaneous fistula
Distal Jejunum (#3)	Female	70	Caucasian	Non-Smoker	Enterocutaneous fistula followed by lap nephrectomy
Distal Ileum (Adjacent to the junction of the ileo-cecal valve)	Female	70	Caucasian	Non-smoker	Right hemicholectomy for displastic polyp removal

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Drug Metabolism and Disposition

The analytical quality control validation for Sodium/Potassium-ATPase (Na/K-ATPase), P-glycoprotein (P-gp) and Breast Cancer Resistance Protein (BCRP) for the University of Manchester has been established and reported previously (Harwood et al., 2015, J Pharm Biomed Anal, 110, 27-33).

Table S2. Analytical quality control validation for Na/K-ATPase by Bertin Pharma.

Protein	**Human Na/K-ATPase**				
MRM channels	**Theoretical injected amount (injected fmol)**	**Mean (injected fmol)**	**SD**	**CV%**	**Accuracy%**
Standard 1	5	5.1	0.2	4.0	103
Standard 2	10	9.4	0.8	8.7	94
Standard 3	50	50.8	1.6	3.1	102
Standard 4	100	95.9	1.9	1.9	96
Standard 5	500	495.8	12.2	2.5	99
Standard 6	1000	1006.2	50	5.0	101
Standard 7	2500	2489.3	72.4	2.9	100
Standard 8	5000	5302.1	198.1	3.7	106
QC LOW 1	10	9.4	0.8	8.3	94
QC MEDIUM 1	200	209.1	8.4	4.0	105
QC LOW 2	10	9.9	0.6	6.5	99
QC HIGH 1	4000	4111.5	138.2	3.4	103
QC MEDIUM 2	200	210.1	6.8	3.2	105
QC HIGH 2	4000	4075.3	131.7	3.2	102

The linearity of the calibration curve was $R^2 = 0.999$. The acceptance criterion for accuracy is 80-120%.

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Drug Metabolism and Disposition

Table S3. Analytical quality control validation for P-gp by Bertin Pharma.

Protein	**Human P-gp**				
MRM channels	**Theoretical injected amount (injected fmol)**	**Mean (injected fmol)**	**SD**	**CV%**	**Accuracy%**
Standard 1	5	5.5	NA	NA	110
Standard 2	10	9.1	1.2	13.3	91
Standard 3	50	46.9	1.3	2.8	94
Standard 4	100	99.6	4.6	4.6	100
Standard 5	500	525.7	6	1.1	105
Standard 6	1000	1029.2	14.3	1.4	103
Standard 7	2500	2491.4	38.7	1.6	100
Standard 8	5000	5148.9	150.2	2.9	103
QC LOW 1	10	9.4	0.9	9.0	94
QC MEDIUM 1	200	194.0	0.8	0.4	97
QC LOW 2	10	10.3	1	9.6	103
QC HIGH 1	4000	3969.7	108.3	2.7	99
QC MEDIUM 2	200	194.2	4.1	2.1	97
QC HIGH 2	4000	3967.1	81.7	2.1	99

The linearity of the calibration curve was $R^2 = 1.0$. The acceptance criterion for accuracy is 80-120%.

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Drug Metabolism and Disposition

Table S4. Analytical quality control validation for BCRP by Bertin Pharma.

Protein	**Human BCRP**				
MRM channels	**Theoretical injected amount (injected fmol)**	**Mean (injected fmol)**	**SD**	**CV%**	**Accuracy%**
Standard 1	5	5.3	NA	NA	106
Standard 2	10	9.5	1	10.3	95
Standard 3	50	NA	NA	NA	NA
Standard 4	100	80.9	1.3	1.6	81
Standard 5	500	478.2	10.1	2.1	96
Standard 6	1000	959.9	33.3	3.5	96
Standard 7	2500	2771.6	116.2	4.2	111
Standard 8	5000	5643.0	230.2	4.1	113
QC LOW 1	10	9.7	2	20.5	97
QC MEDIUM 1	200	142.5	6.7	4.7	71
QC LOW 2	10	9.4	0.3	3.0	94
QC HIGH 1	4000	4446.1	213	4.8	111
QC MEDIUM 2	200	146.7	5.8	3.9	73
QC HIGH 2	4000	4494.2	232.3	5.2	112

The linearity of the calibration curve was $R^2 = 0.999$. * rejected value outside of the acceptance criterion (80-120%)

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MGKGVGRDKYEPAAVSEQGDKKGKKGKKDRDMDELKKEVSMDDHKLSLDELHRKYGTDL
SRGLTSARAAEILARDGPNALTPPPTTPEWIKFCRQLFGGFSMLLWIGAILCFLAYSIQAATE
EEPQNDNLYLGVVLSAVVIITGCFSYYQEAKSSKIMESFKNMVPQQALVIRNGEKMSINAEE
VVVGDLVEVKGGDRIPADLRIISANGCKVDNSSLTGESEPQTRSPDFTNENPLETRNIAFFS
TNCVEGTARGIVVYTGDRTVMGRIATLASGLEGGQTPIAAEIEHFIHIITGVAVFLGVSFFILSL
ILEYTWLEAVIFLIGIIVANVPEGLLATVTVCLTLTAKRMARKNCLVKNLEAVETLGSTSTICSD
KTGTLTQNRMTVAHMWFDNQIHEADTTENQSGVSFDKTSATWLALSRIAGLCNRAVFQAN
QENLPILKRAVAGDASESALLKCIELCCGSVKEMRERYAK**IVEIPFNSTNK**YQLSIHKNPNTS
EPQHLLVMKGAPERILDRCSSILLHGKEQPLDEELKDAFQNAYLELGGLGERVLGFCHLFLP
DEQFPEGFQFDTDDVNFPIDNLCFVGLISMIDPPR**AAVPDAVGK**CRSAGIKVIMVTGDHPIT
AKAIAKGVGIISEGNETVEDIAARLNIPVSQVNPRDAKACVVHGSDLKDMTSEQLDDILKYHT
EIVFARTSPQQKLIIVEGCQRQGAIVAVTGDGVNDSPALKKADIGVAMGIAGSDVSKQAADM
ILLDDNFASIVTGVEEGRLIFDNLKKSIAYTLTSNIPEITPFLIFIIANIPLPLGTVTILCIDLGTDMV
PAISLAYEQAESDIMKRQPRNPKTDKLVNERLISMAYGQIGMIQALGGFFTYFVILAENGFLPI
HLLGLRVDWDDRWINDVEDSYGQQWTYEQRKIVEFTCHTAFFVSIVVVQWADLVICKTRRN
SVFQQGMKNKILIFGLFEETALAAFLSYCPGMGVALRMYPLKPTWWFCAFPYSLLIFVYDEV
RKLIIRRRPGGWVEKETYY

Figure S1. Na/K-ATPase amino acid sequence highlighting the BPh selected peptide in bold and underlined text and UoM peptide in bold text.

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MDLEGDRNGGAKKKNFFKLNNKSEKDKKEKKPTVSVFSMFRYSNWLDKLYMVVGTLAAIIH
GAGLPLMMLVFGEMTDIFANAGNLEDLMSNITNRSDINDTGFFMNLEEDMTRYAYYYSGIG
AGVLVAAYIQVSFWCLAAGRQIHKIRKQFFHAIMRQEIGWFDVHDVGELNTRLTDDVSKINE
GIGDKIGMFFQSMATFFTGFIVGFTRGWKLTLVILAISPVLGLSAAVWAKILSSFTDKELLAYA
K**AGAVAEEVLAA**I**R**TVIAFGGQKKELERYNKNLEEAKRIGIKKAITANISIGAAFLLIYASYALA
FWYGTTLVLSGEYSIGQVLTVFFSVLIGAFSVGQASPSIEAFANARGAAYEIFKIIDNKPSIDS
YSKSGHKPDNIKGNLEFRNVHFSYPSRKEVKILKGLNLKVQSGQTVALVGNSGCGKSTTVQ
LMQRLYDPTEGMVSVDGQDIRTINVRFLREIIGVVSQEPVLFATTIAENIRYGRENVTMDEIE
KAVKEANAYDFIMKLPHKFDTLVGERGAQLSGGQKQRIAIARALVRNPKILLLDEATSALDTE
SEAVVQVALDKARKGRTTIVIAHRLSTVRNADVIAGFDDGVIVEKGNHDELMKEKGIYFKLVT
MQTAGNEVELENAADESKSEIDALEMSSNDSRSSLIRKRSTRRSVRGSQAQDRKLSTKEAL
DESIPPVSFWRIMKLNLTEWPYFVVGVFCAIINGGLQPAFAIIFSKIIGVFTRIDDPETKRQNSN
LFSLLFLALGIISFITFFLQGFTFGKAGEILTKRLRYMVFRSMLRQDVSWFDDPKNTTGALTTR
LANDAAQVKGAIGSRLAVITQNIANLGTGIIISFIYGWQLTLLLLAIVPIIAIAGVVEMKMLSGQA
LKDKKELEGSGKIATEAIENFRTVVSLTQEQKFEHMYAQSLQVPYRNSLRKAHIFGITFSFTQ
AMMYFSYAGCFRFGAYLVAHKLMSFEDVLLVFSAVVFGAMAVGQVSSFAPDYAKAKISAAH
IIMIIEKTPLIDSYSTEGLMPNTLEGNVTFGEVVFNYPTRPDIPVLQGLSLEVKKGQTLALVGS
SGCGKSTVVQLLER**FYDPLAGK**VLLDGKEIKRLNVQWLRAHLGIVSQEPILFDCSIANIAYGD
NSRVVSQEEIVRAAKEANIHAFIESLPNKYSTKVGDKGTQLSGGQKQRIAIARALVRQPHILL
LDEATSALDTESEKVVQEALDKAREGRTCIVIAHRLSTIQNADLIVVFQNGRVKEHGTHQQLL
AQKGIYFSMVSVQAGTKRQ

Figure S2. P-gp amino acid sequence highlighting the BPh selected peptide in bold and underlined text and UoM peptide in bold text. The single nucleotide polymorphism (SNP) present at position 261 (c.I261V – p.A781G) in the UoM selection is in gray text.

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MSSSNVEVFIPVSQGNTNGFPATASNDLKAFTEGAVLSFHNICYRVKLKSGFLPCRKPVEKE

ILSNINGIMKPGLNAILGPTGGGK**SSLLDVLAAR**KDPSGLSGDVLINGAPRPANFKCNSGYV

VQDDVVMGTLTVRENLQFSAALRLATTMTNHEKNERINR**VIQELGLDK**VADSKVGTQFIRG

VSGGERKRTSIGMELITDPSILFLDEPTTGLDSSTANAVLLLLKRMSKQGRTIIFSIHQPRYSIF

KLFDSLTLLASGRLMFHGPAQEALGYFESAGYHCEAYNNPADFFLDIINGDSTAVALNREED

FKATEIIEPSKQDKPLIEKLAEIYVNSSFYKETKAELHQLSGGEKKKKITVFKEISYTTSFCHQL

RWVSKRSFKNLLGNPQASIAQIIVTVVLGLVIGAIYFGLKNDSTGIQNRAGVLFFLTTNQCFSS

VSAVELFVVEKKLFIHEYISGYYRVSSYFLGKLLSDLLPMRMLPSIIFTCIVYFMLGLKPKADA

FFVMMFTLMMVAYSASSMALAIAAGQSVVSVATLLMTICFVFMMIFSGLLVNLTTIASWLSW

LQYFSIPRYGFTALQHNEFLGQNFCPGLNATGNNPCNYATCTGEEYLVKQGIDLSPWGLW

KNHVALACMIVIFLTIAYLKLLFLKKYS

Figure S3. BCRP amino acid sequence highlighting the BPh selected peptide in bold and underlined text and UoM peptide in bold text.