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Absolute immunoquantification of the expression of ABC transporters P-glycoprotein, breast cancer resistance protein and multidrug resistance-associated protein 2 in human liver and duodenum

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ABSTRACT

The ATP-binding cassette (ABC) transporters breast cancer resistance protein (BCRP), multidrug resistance-associated protein 2 (MRP2), and P-glycoprotein (Pgp) are important in the distribution and elimination of many drugs and endogenous metabolites. Due to their membrane location and hydrophobicity it is difficult to generate purified protein standards to quantify these transporters in human tissues. The present study generated transporter proteins fused with the S-peptide of ribonuclease for use as standards in immunoquantification in human liver and small intestine. Quantification of the S•tag™, a 15 amino acid peptide, is based on the formation of a functional ribonuclease activity upon its high affinity reconstitution with ribonuclease S-protein. S-tagged transporters were used as full-length protein standards in the immunoquantification of endogenous BCRP, MRP2, and Pgp levels in 14 duodenum and 13 liver human tissue samples. Expression levels in the duodenum were 305 ± 248 (BCRP), 66 ± 70 (MRP2), and 275 ± 205 (Pgp) fmoles per cm^2. Hepatic levels were 2.6 ± 0.9 (BCRP), 19.8 ± 10.5 (MRP2), and 26.1 ± 10.1 (total Pgp) pmoles per g of liver. The mean hepatic scaling factor was 35.8 mg crude membrane per g of liver, and the mean duodenal scaling factor was 1.3 mg crude membrane per cm^2 mucosal lining. Interindividual variability was greater in duodenal samples than liver samples. It is hoped that this innovative method of quantifying these transporters (and other membrane proteins) will improve *in vivo–in vitro* extrapolation and *in silico* prediction of drug absorption and elimination, thus supporting drug development.

1. Introduction

The ATP-binding cassette (ABC) transporters breast cancer resistance protein (BCRP, ABCG2), multidrug resistance-associated protein 2 (MRP2, ABCC2), and P-glycoprotein (Pgp, ABCB1) use ATP hydrolysis to drive the export of a huge range of structurally diverse compounds from cells and are important in the absorption, distribution, metabolism and elimination of xenobiotics and endogenous chemicals [1–4]. Expression of BCRP, MRP2, and Pgp at the apical side of enterocytes and hepatocytes means that they are ideally situated to restrict absorption of dietary toxins and drugs from the gut, and to eliminate compounds from the liver into the bile [5,6]. Expression levels of these transporters have a profound effect on individual response to drugs and other xenobiotics.

Computer-based methods predicting pharmacokinetics, pharmacodynamics and drug–drug interactions are important tools supporting drug development [7,8]. Their viability is dependent, among other factors, on the availability of high quality data on the expression, distribution, variability of drug metabolizing enzymes, transporters, etc. In contrast to drug metabolizing enzymes such as cytochromes P450 [9,10] and sulfotransferases [11], there is little information on the protein expression levels of ABC transporters. This is mainly because transporters, being integral membrane proteins, are extremely difficult to purify. Purification and reconstitution into proteoliposomes has been achieved (e.g. [12]), but this is expensive and technically challenging. Thus studies investigating transporter protein expression levels in solid tissues in particular have been comparative at best. Proteomic approaches [13] have been used to determine protein expression levels of trypsin-digested BCRP

Abbreviations: BCRP, breast cancer resistance protein; MRP2, multidrug resistance-associated protein 2; Pgp, P-glycoprotein.

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and MRP2 in tissue samples [14–16], however these methods have a number of limitations, including the reproducibility of extraction and sample digestion. Other approaches to quantification, for example flow cytometry, have been taken using cells such as bone marrow (e.g. [17,18]), but these are not applicable to solid tissues. A straightforward method for the production and quantification of full-length protein standards would therefore be an important contribution to the study of ABC transporters.

Here we present the application of a novel approach to this problem recently devised in our laboratory for quantification of UDP-glucuronosyltransferases [19] using the S-tag/S-protein system [20] to generate transporter protein standards for immunoquantification. Cloning and expression of cDNAs encoding these transporters as fusion proteins with the 15 amino acid S•tag™ peptide allowed the recombinant transporters to be quantified using the fact that S•tag™ and S-protein combine with high affinity and efficiency to reconstitute a functional ribonuclease enzyme whose activity can be determined using a sensitive spectrophotometric assay. The amount of S•tag™ present can thus be quantified which in turn infers the quantity of S-tagged transporter in the preparation. The quantified recombinant S-tagged transporters were then used to generate standard curves for immunoquantification in human duodenum and liver. This represents the first comprehensive analysis of protein expression levels of these transporters in human tissue using an immunochemical approach.

2. Materials and methods

2.1. Cloning and expression of S-tagged transporters

S-tagged BCRP, MRP2, and Pgp were cloned by PCR, using pET32b (Novagen/Merck, Darmstadt, Germany), IMAGE clone 100004938 (Source BioScience, Nottingham, UK), pcDNA3.1 harbouring MRP2 (kindly provided by Professor D. Keppler, German Cancer Research Centre, Heidelberg, Germany), and IMAGE clone 40146455 (Source BioScience) as templates for S-tag, BCRP (GI:123982985), MRP2 (GI:188595701), and Pgp (GI:120660210), respectively. The sequence encoding the 15 amino acid S-tag was cloned at either the 5′ or the 3′ end of the transporter cDNA. PCR products consisting of the S-tagged transporter cDNA were subcloned into either pSC-A or pSC-B (Invitrogen, Paisley, UK), before being digested and ligated into pcDNA5/FRT/TO (Invitrogen).

Each pcDNA5/FRT/TO construct encoding either an S-tagged or untagged transporter was co-transfected with the vector pOG44, encoding the Flp recombinase required for targeted integration, into FT293 cells (a modified HEK293 cell line) using lipofectamine2000 (Invitrogen). Cells were selected and maintained in hygromycin selection media (HSM) consisting of DMEM supplemented with 10% fetal bovine serum, 100 units/mL penicillin, 100 μg/mL streptomycin, 2 mM L-glutamine, 75 μg/mL hygromycin and 15 μg/mL blasticidin. Expression of the recombinant protein was derepressed by culturing cells in HSM supplemented with 0.5 μg/mL tetracycline for 48 h.

2.2. Preparation and quantification of protein standards

Cells expressing the recombinant protein were harvested, washed twice in PBS, and resuspended in sucrose HEPES buffer (SHR – 0.25 M sucrose, 10 mM HEPES pH 7.4, plus complete protease inhibitor cocktail (Roche, Burgess Hill, UK)). Cells were sonicated, and the lysate centrifuged at 1000 × *g* for 5 min at 4 °C. The pellet containing nuclei and whole cell debris was discarded, and the supernatant centrifuged at 100,000 × *g* for 1 h at 4 °C. The pellet was resuspended in SHR using a tight fit glass homogeniser for 30 strokes, and the non-ionic detergent Lubrol-PX (Sigma–Aldrich, Gillingham, UK) added to a final concentration of 1% (w/v). Samples were sonicated and incubated on ice for 1–3 h before centrifuging at 100,000 × *g* for 1 h at 4 °C. The supernatant, containing the soluble fraction, was subjected to the S•tag™ Rapid Assay and used as standard in immunoquantification.

2.3. S•tag™ Rapid Assay

The S•tag™ Rapid Assay Kit (Novagen/Merck) was adapted for 0.5 mL microcentrifuge tubes. Assays comprised, in a total volume of 195 μL, 20–50 μg of either S-tagged transporter or untagged transporter (for blanks) and assay buffer (20 mM Tris–HCl pH 7.5, 100 mM NaCl, 100 μg/mL poly(C)). 5 μL of S-protein (0.01 pmol/μL) were added to each reaction tube, vortexed and incubated at 37 °C for 5 min. 50 μL ice-cold 25% (w/v) tricholoroacetic acid were added and the samples vortexed and cooled on ice for 5 min. Samples were then centrifuged at 14,000 × *g* for 10 min and 200 μL of the supernatant transferred to a well of a 96-well UV plate and the absorbance at 280 nm read on a microplate spectrophotometer, against the known S-peptide standard assayed in an identical manner.

2.4. Human liver and duodenal samples

Human duodenum samples were obtained from the UK Human Tissue Bank (DeMontfort University, Leicester, UK), and human liver samples were as reported previously [11,21,22]. Ethical approval for local use of samples was obtained from the Tayside Medical Research Ethics Committee. 4 g sections of duodenum were scraped to remove the mucosal lining, which was then homogenised with SHR in a glass Dounce homogeniser with Teflon mortar, for up to 5 min. 0.5 g samples of human liver were homogenised with SHR in a glass Dounce homogeniser with Teflon mortar, for up to 5 min, and any cartilaginous tissue or other unhomogenised material was discarded. Homogenates were centrifuged at 1000 × *g* for 3 min at 4 °C and the supernatant was then centrifuged at 100,000 × *g* for 1 h at 4 °C. The pellet containing the crude membrane extract was resuspended in SHR using a tight fitting glass Dounce homogeniser. Scaling factors for intestinal samples were based on the surface areas of the mucosal linings, which were measured by placing the thawed sample on laminated 4 mm^2 graph paper and counting the number of squares. The mean (±SD) scaling factor for the duodenal samples was 1344 ± 737 μg/cm^2. For the liver samples, the scaling factors were based on the recovery of total protein in the crude membrane fraction related to the weight of the wet tissue and the mean (±SD) scaling factor was 35.8 ± 14.3 mg/g.

2.5. Immunoblotting

Crude membrane extracts prepared from human tissues, and serially diluted standards comprising quantified recombinant S-tagged transporters, were fractionated on precast 4–12% Bis-Tris SDS-PAGE gels in MOPS buffer according to the manufacturer's instructions (Invitrogen). Proteins were transferred to PVDF membranes (Millipore, Watford, UK) in a large wet transfer tank for 2 h at 100 V [23], in transfer buffer comprising 192 mM glycine, 25 mM Tris, 0.04% (w/v) SDS, and 20% (v/v) methanol. Gels were stained with Coomassie blue to ensure complete transfer of proteins; gels were routinely seen to be clear, suggesting efficient transfer of standards and test samples. Following transfer membranes were exposed to blocking buffer (5% (w/v) skimmed milk powder in TBS-T (50 mM Tris, 150 mM NaCl, and 0.05% (v/v) Tween-20)) and subjected to immunostaining.

For immunoquantification of MRP2, PVDF membranes were blocked for 1 h in blocking buffer, and then incubated overnight at 4 °C with the primary antibody M_2III-5 (Alexis/Enzo Life Sciences, Exeter, UK), diluted 1:500 in blocking buffer. They were then washed three times in blocking buffer, incubated with secondary antibody (1:1000 HRP-conjugated rabbit-anti-mouse IgG (DAKO, Ely, UK) in blocking buffer) for 1–2 h, washed twice in blocking buffer, and once in TBS-T. For BCRP and Pgp, the PVDF membranes were blocked for 1 h in milk blocking buffer (5% (w/v) skimmed milk powder in PBS-T (PBS supplemented with 0.05% (v/v) Tween-20)) followed by three 5 min washes in PBS-T, and 30 min incubation with BSA blocking buffer (1% (w/v) BSA in PBS-T). The membranes were incubated overnight at 4 °C with either primary antibody C219 (Calbiochem/Merck) diluted 1:100 for Pgp, or BXP21 (Sigma–Aldrich) diluted 1:200 for BCRP (both in BSA blocking buffer). Following three washes with PBS-T, they were incubated with secondary antibody (1:1000 HRP-conjugated rabbit anti-mouse IgG (DAKO) in BSA blocking buffer) for 1–2 h, and washed three times in PBS-T. Membranes were incubated with ECL reagent and exposed to film for a variety of exposure lengths to ensure no saturation of the bands.

2.6. Quantification

Films exposed to immunoblots were scanned, using a standard office scanner, and the band densities measured using Quantiscan

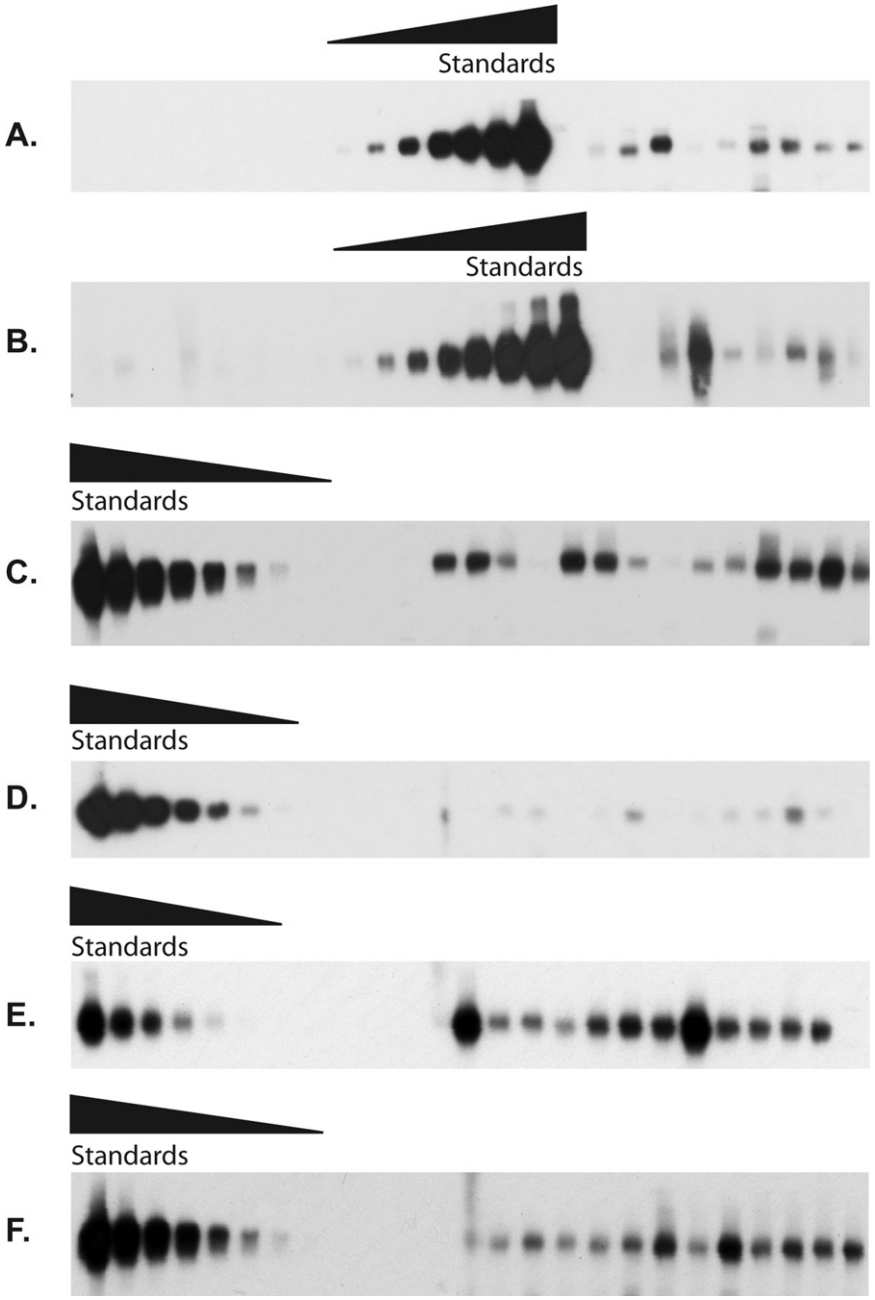

Fig. 1. Representative immunoblots for each transporter in human duodenum and liver tissue samples. Lanes were loaded with a serial dilution of recombinant S-tagged transporter as the protein standard (between 0.5 and 84.7 fmol for BCRP, 0.3 and 49.1 fmol for MRP2, and 1.0 and 162.1 fmol for Pgp). Other lanes contain crude membrane extracts prepared from human liver and duodenum samples, typically between 12 and 123 μg total protein of crude membrane preparation was loaded per lane. A – duodenum/BCRP, B – duodenum/MRP2, C – duodenum/Pgp, D – liver/BCRP, E – liver/MRP2, and F – liver/Pgp.

software (Biosoft, Cambridge, UK). Endogenous transporter expression in the human tissue samples was calculated by comparing the band densities of samples with a standard curve derived from the band densities of the recombinant S-tagged transporter standards. Each sample was blotted eight times with each antibody, using band density values within the linear range of the relevant standard curves.

3. Results

3.1. Characterisation of recombinant transporter proteins

Recombinant BCRP, MRP2, and Pgp generated with no tag, or with the S-tag placed at the N- or C-terminus were characterised using a combination of immunofluorescence microscopy, immunoblotting, and transport assays (full details will be published elsewhere). We found C-terminally tagged BCRP to be mislocalised to the endoplasmic reticulum with no expression at the plasma membrane, however with the S-tag at the N-terminus BCRP was found predominantly at the plasma membrane and was able to extrude the BCRP substrates H33342 and mitoxantrone. Both N- and C-terminally S-tagged MRP2 localised predominantly at the plasma membrane, although the S-tag appeared less stable when placed at the N-terminus. Both N- and C-terminally S-tagged Pgp were localised predominantly to the plasma membrane, and were able to extrude the Pgp substrate H33342 from whole cells. Due to the similarity of their behaviour with untagged protein, recombinant N-terminally S-tagged BCRP and Pgp, and C-terminally S-tagged MRP2 were chosen for use as full-length protein standards in immunoquantification.

For both liver and duodenum, and for each of the three antibodies used, immunoblotting conditions were optimised such that the resulting bands were within the linear range of the standard curves generated from the purified recombinant S-tagged transporters. Fig. 1 shows a representative blot for each tissue and antiserum used in this study.

3.2. Expression of BCRP, MRP2, and Pgp in human tissue samples

The expression levels of BCRP, MRP2, and Pgp in 13 liver and 12 duodenum samples were investigated and calculated as attomoles (1×10^{-18}) per μg of total protein in the crude membrane preparation (Table 1). In order to present these data in a form useful to other researchers, we also calculated scaling factors enabling the expression levels per g of liver and per cm^2 of duodenum to be considered. Scaling factors were based on starting material of the liver and duodenal tissue samples, whilst taking into account loss of sample during sample preparation; for example sections that were not completely homogenised, where further processing may have resulted in heat degradation of proteins. We calculated the scaling factors to be 1.3 mg crude membrane extract per cm^2 of duodenal mucosal lining, and 35.8 mg crude membrane extract per g of liver tissue. The results given in Table 1 were calculated using the mean scaling factor for all samples.

We found considerable differences in the expression levels of the three transporters between the liver and duodenum, and there was also considerable interindividual variation in the expression of these proteins (Table 1). BCRP was expressed at 3-fold higher levels in the duodenum than in the liver (on a per μg total protein basis), whereas MRP2 and Pgp were both expressed at higher levels in the liver (11.3-fold and 3.5-fold, respectively). Pgp was the major expressed transporter in liver, with BCRP the lowest represented (10-fold less protein than Pgp), whereas in the duodenum samples MRP2 exhibited the lowest expression, with BCRP and Pgp present at similar quantities (approximately 4-fold higher than MRP2). In both liver and duodenum, MRP2 appeared to show the highest levels of interindividual variation with a fold difference of 7.4 and 40.2 respectively. In the duodenum the fold difference may have been exaggerated due to the low level of expression, even though only bands within the linear range of the standard curve were used. In addition, the interindividual variation appeared to be higher in the gut samples than in the liver samples. Fig. 2 shows the relative expression levels of the 3 transporters by normalizing against the expression level in either the liver (A) or intestine (B).

The expression levels and relative rank order of expression of BCRP, MRP2, and Pgp in liver and intestine were compared with values published by other groups based on expression at mRNA levels (Fig. 3). Overall, there was not a strong relationship between our protein expression levels and those reported by other groups based on mRNA levels. In the intestine, BCRP was generally found to be the most abundant of the three transporters as we have reported here, however in the liver our study showed Pgp to be the most abundant whereas the study by Hilgendorf et al. showed MRP2 to be significantly more abundant than Pgp [24].

We found no statistically significant correlations between sex and transporter expression, however we did find a significant positive correlation of hepatic Pgp levels with age (Pearson's correlation coefficient $r = 0.75$, $P < 0.01$).

Table 1
Expression of BCRP, MRP2, and Pgp in human duodenum and liver samples.

	BCRP	MRP2	Pgp
Duodenum ($n = 14$)			
Attomoles per μg total protein			
Mean ± SD	227 ± 184	49 ± 52	205 ± 153
Range (fold diff)	49–593 (12x)	4–159 (40x)	91–650 (7x)
Femtomoles per cm^2 of mucosal lining (using mean scaling factors)			
Mean ± SD	305 ± 248	66 ± 70	275 ± 205
Range (fold diff)	66–796 (12x)	5–214 (43x)	122–873 (7x)
Liver ($n = 13$)			
Attomoles per μg total protein			
Mean ± SD	73 ± 24	554 ± 294	727 ± 282
Range (fold diff)	45–141 (3x)	171–1268 (7x)	303–1285 (4x)
Femtomoles per g of liver (using mean scaling factor)			
Mean ± SD	2605 ± 877	19 839 ± 10 518	26 054 ± 10 105
Range (fold diff)	1622–5035 (3x)	6141–45 433 (7x)	10 865–46 058 (4x)

Each liver and duodenum sample was analysed on 8 separate immunoblots.

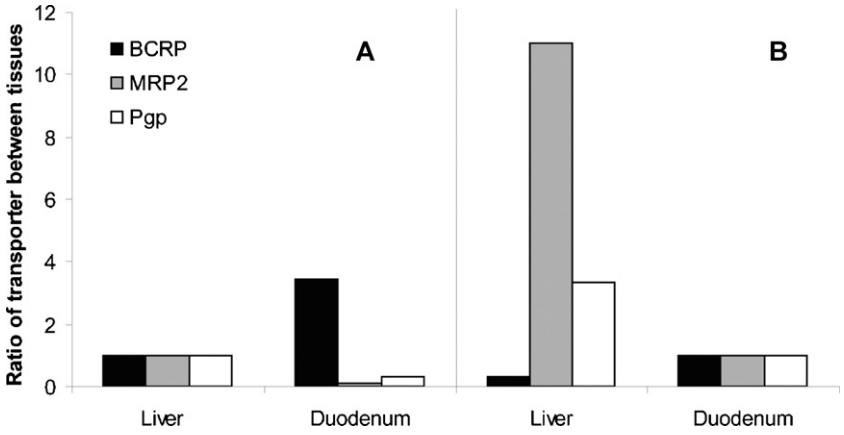

Fig. 2. Comparison of transporter expression values in liver and duodenum tissue. Mean expression level data are expressed relative to the mean values obtained in the membranes prepared from either liver (A) or duodenum (B).

4. Discussion

Due to the lack of quantified protein standards, studies determining transporter protein levels have generally been comparative rather than absolute. Here we have applied a novel method to quantify the expression of three important transporter proteins in human liver and intestine using the S-tag/S-protein system. The method is robust and easily applicable to any protein for which good antibodies are available. The principal drawbacks of this method include the dependence on high quality, specific antibodies and the labour intensive processes of gel electrophoresis and immunoblotting. Antibody specificity is another potential issue with the method. The antibody C219, which is very widely used for immunochemical detection of Pgp, is known to display cross-reactivity with a number of other proteins [25] – depending on the level of expression of these in liver and intestinal samples, this may lead to an overestimation of the expression levels of Pgp. However, unlike proteomic approaches it does not require highly specialised and expensive mass spectrometry equipment or expensive labelled peptides [13,26]. Another issue with proteomic quantification is the potential for incomplete digestion of target proteins, particularly in membrane preparations. A recent publication [27] presents a mass spectrometry method for the quantification of a number of transporters (including MRP2 and BCRP) and drug metabolizing enzymes, however the absolute expression values for the proteins are not presented, only data on the reliability and reproducibility of the method. Another potential problem with all of these methods, and in particular of comparisons between them, lies with the preparation methods for the membrane fractions. Here we tested a variety of tissue fractionation, differential centrifugation protocols and solubilisation techniques before identifying the combination that produced the highest recoveries of the proteins under investigation. Other investigators use commercially available "kits" for isolation of membrane fractions that may not be optimised for these particular proteins.

Whilst there are several mRNA studies that have compared BCRP, MRP2, and Pgp expression levels, studies comparing the protein levels are extremely rare; this study, using quantified standards, allowed an absolute value of transporter expression to be derived. Other studies have claimed to be semi-quantitative e.g. [28], but to the best of our knowledge the only other group to quantify the number of transporters in a particular tissue is Lai's group [14,16]. This group used AQUA (absolute quantification) involving tryptic digest of membrane extracts followed by LC–MS to compare the fragment peak with that of a standard (a heavy-label synthetic peptide) to quantify the transporter. Another group [29] has used a method based on a quantitative immunocytofluorometric assay to detect cell surface antigens on whole cells [30], although this assay has the flaw that the efficiency with which the primary antibody binds Pgp is not accounted for, and intracellular pools of Pgp would not be detected, but these pools could contribute to efflux activity when cells are exposed to a Pgp substrate.

Another significant issue in comparing protein expression values in different studies is the way in which samples are prepared and, subsequently, values calculated. As preparation methods can differ considerably it is therefore difficult to compare values, hence our estimation of appropriate hepatic and intestinal scaling factors. When the values in the present method were compared with those determined by AQUA, similar values were seen (liver BCRP levels were 0.07 and 0.15 fmoles per μg of preparation, and MRP2 levels were 0.55 and 0.60 fmoles per μg of preparation, in the present study and the AQUA [15] studies, respectively). However, the present study used a crude membrane preparation by centrifugation, whilst the group using AQUA used a membrane protein extraction kit.

It is difficult to compare the expression of a particular transporter between the gut and liver samples because the tissues and preparation methods are different. In terms of the transporter level per μg of sample preparation (Table 1), the gut contains 332%, 9%, and 31% of the BCRP, MRP2, and Pgp found in the liver, respectively. Studies comparing human mRNA in the liver and gut have also found lower levels of BCRP in the liver compared to the gut [24,31], though the difference was less pronounced than in the present study. MRP2 has been reported to have similar [24], higher [31], or much higher [32] expression in the liver compared to the gut, which generally agrees with the present study in which MRP2 levels were 11-fold higher in the liver per μg of crude membrane extract (Fig. 3). Pgp mRNA expression has been reported to be similar [24] or lower [31] in the liver than the gut, whereas in the present study Pgp protein levels were over 3-fold higher in the liver than the gut per μg of crude membrane extract (Fig. 2).

The present study found a large interindividual variability which concurs with other studies. A 6-fold difference in MRP2 protein expression levels was seen in human jejunum samples [15], another study reported considerable interindividual variation in BCRP, MRP2, and Pgp, particularly at the protein level [33]. A study investigating mRNA levels in human jejunum samples found variations in BCRP, MRP2, and Pgp of 35.4, 50, and 40.4%, respectively, 66% of which was predicted to be down to

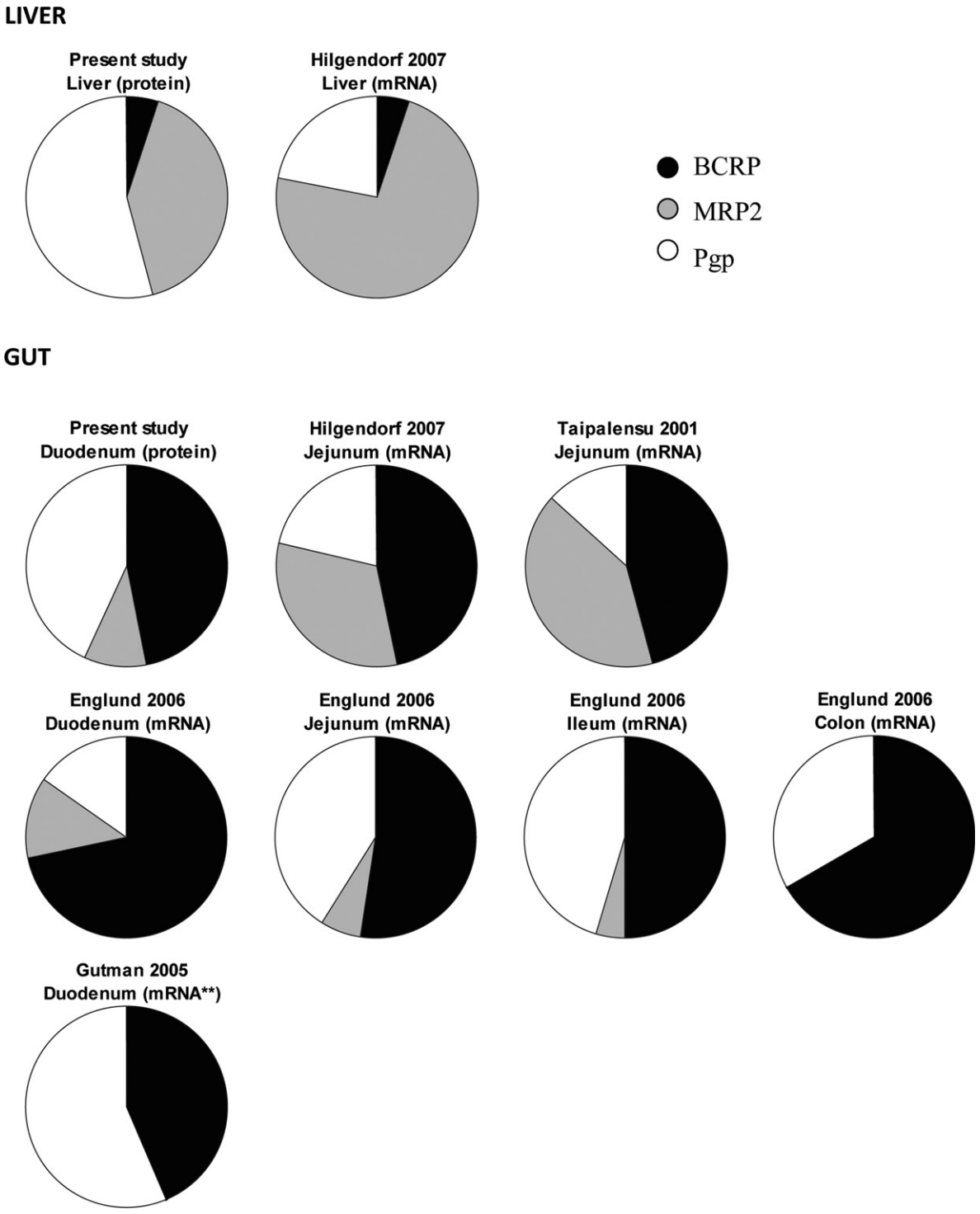

Fig. 3. Summary of relative expression of BCRP, MRP2, and Pgp determined by various studies. Mean expression values for each transporter are displayed as percentages of the sum of the three immunoquantified transporter. **MRP2 mRNA level was not determined for duodenal samples in [37].

interindividual differences in gene expression [34]. A study of human duodenum, jejunum, ileum, and colon reported a 2–5-fold interindividual variation in BCRP, MRP2, and Pgp mRNA levels within each section [35]. Pgp levels in 91 human duodenal biopsies have been reported to show a 10-fold difference in levels between individuals [36]. Some studies do not comment on interindividual variation, but graphs presented show both low [37] and high [28] levels of interindividual variation. Genetic, epigenetic, and environmental factors can affect the expression levels of BCRP, MRP2, and Pgp, and it is interesting to note that Han–Wistar rats show considerable interindividual variation in ABC transporter mRNA levels in the gut, despite the animals being relatively genetically homogenous, and housed under controlled conditions. The authors also observed intraindividual variation which could be attributed to sections of the gut experiencing different exposures to digesting food, leading to different transport requirements [38]. In the present study, there appeared to be a higher interindividual variability in ABC transporter protein expression levels in the

duodenum samples than in the liver samples, which agrees with a study that found interindividual variation in ABC transporter and PXR mRNA levels were higher in the intestine than the liver [39].

The present study shows for the first time a novel approach to generating full length standards for immunoquantification of BCRP, MRP2, and Pgp expression levels in human liver and duodenum samples. The information presented will also prove a valuable addition to the datasets available in support of *in silico* pharmacokinetic/pharmacodynamic modelling tools such as Simcyp (www.simcyp.com).

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