# A Cross-Laboratory Comparison of Caco-2 and Human Intestinal Drug Transporter Protein Abundances

Matthew D Harwood[†‡], Brahim Achour[†], Yann Courbebaisse[#], Matthew R Russell[†], Sibylle Neuhoff[‡], Gordon Carlson[†], Geoffrey Warhurst[†], Amin Rostami-Hodjegan[†‡]

[†] The University of Manchester & Salford Royal Hospital Trust, Manchester, UK.
[‡] Simcyp Ltd (a Certara Company), Sheffield, UK
[#] Bertin Pharma, Orleans, France

**Introduction:** Numerous laboratories utilising a diverse range of techniques are quantifying the absolute abundance of drug transporter proteins by Quantitative Targeted Absolute Proteomic (QTAP) strategies in mammalian tissues and *in vitro* cell systems. Ten-fold differences in absolute abundances have been observed for specific transporter isoforms in non-matched samples between laboratories, for example, hepatic OATP1B1[1,2]. Ascertaining if differences in abundances are derived from intrinsic biological variability, or variability associated with assay-specific techniques, and/or specific data analysis within each laboratory, are crucial for generating robust PBPK models that reflect *in vivo* abundances. Therefore, a multi-centre study evaluating the consistency and comparability of the preparation steps and analytic outcome has been advocated[3]. Caco-2 and human intestinal membrane fractions were prepared. This study then assessed the comparability in absolute transporter abundances for 3 proteins; Sodium/Potassium-ATPase (Na/K-ATPase), P-glycoprotein (P-gp) and Breast Cancer Resistance Protein (BCRP) in membrane fractions, within 2 independent laboratories, **The University of Manchester (UoM)**, Manchester, UK and **Bertin Pharma (BPh)**, Orleans, France .

**Methods:** Caco-2 cells (ATCC-HTB-37) passage 25-35 (n=8) or passage 111 were grown for 10, 21 and, 29 days on 44-cm$^2$ Transwell filters (0.4 µM pore size).

Macroscopically normal human distal jejunum (DJ, n=3) and distal ileum (DJ, n=1) were obtained immediately after resection from patients undergoing elective surgery at Salford Royal Hospital under consent (REC 12/NW 0306).

Caco-2 cells scrape harvested from filters and fresh human intestinal enterocytes harvested by elution using an EDTA-chelation method, underwent a differential centrifugation procedure to obtain total or plasma membrane fractions and were stored at -80ºC. Protein content was subsequently determined by BCA assay. Membrane proteins (typically >50 µg) were either shipped on dry ice to BPh or retained for analysis at UoM.

### The University of Manchester

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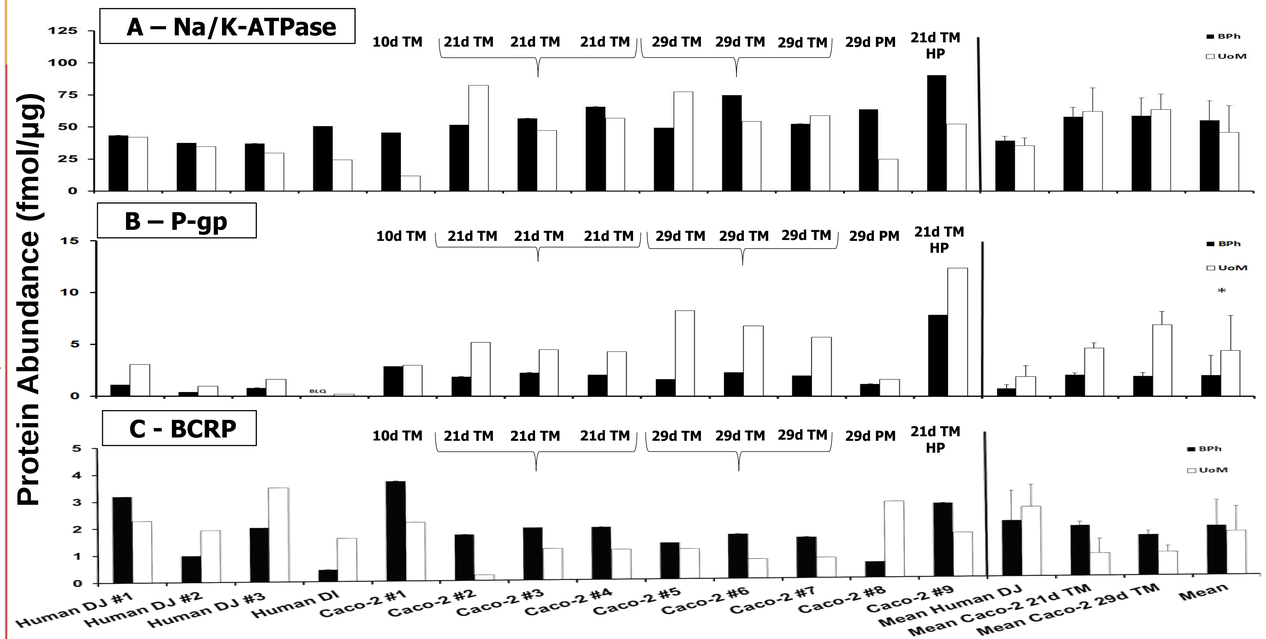
|---|---|
| Peptide Selection | • *In Silico* – UoM – ConSEQUENCE program |
| Standard Generation | • Quantitative Concatenation (QconCAT)[4] |
| Selected Peptides | • Na/K-ATPase – IVEIPFNSTN[**K**$^{13}$C]<br>• P-gp – AGAVAEEVLAAI[**R**$^{13}$C]<br>• BCRP - VIQELGLD[**K**$^{13}$C] |
| Digestion | • Deoxycholate denaturation, Lys-C + trypsin digest |
| LC-MS/MS | • Nano flow LC – nanoAcquity (Waters) with TSQ Vantage (Thermo), selected reaction monitoring |

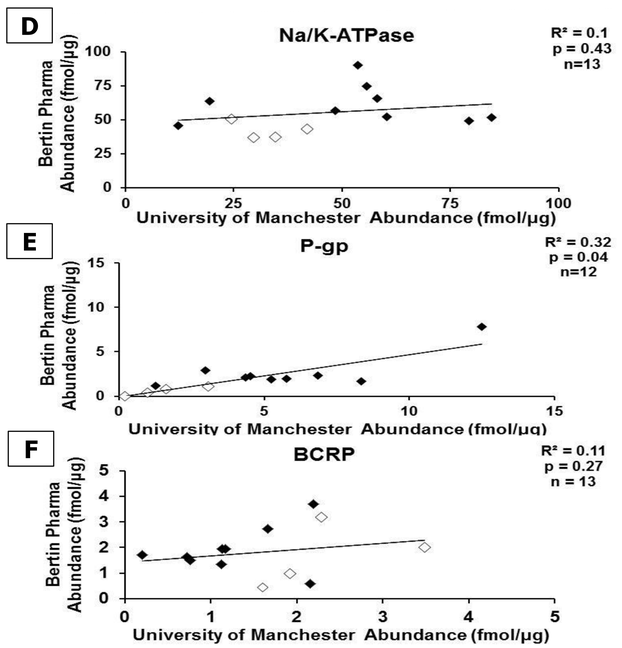
### Bertin Pharma

| | |
|---|---|
| Peptide Selection | • *In Silico* - Tohoku University – Professor Tetsuya Terasaki |
| Standard Generation | • Absolute Quantification (AQUA)[5] |
| Selected Peptides[6] | • Na/K-ATPase - AAVPDA[**V**$^{13}$C,$^{15}$N]GK<br>• P-gp - FYDPL[**A**$^{13}$C,$^{15}$N]GK<br>• BCRP - SSL[**L**$^{13}$C,$^{15}$N]DVLAAR |
| Digestion | • MS2Plex-based process including trypsin-based digest |
| LC-MS/MS | • Normal flow LC – Flexar LC (Perkin Elmer) with API5500 (AB Sciex), selected reaction monitoring |

The native to standard peak area under the curve ratio for ≥ 2 selected transitions for each peptide was used to calculate absolute protein abundances in femtomol (fmol) per µg (fmol/µg) membrane protein.

**Results:** Quality control – Peptide linearity, precision (CV≤15%) and quantification limits (LLOQ) were determined – LLOQ BPh-0.125 fmol/µg, UoM-0.2 fmol/µg




**Na/K-ATPase** (a basal membrane marker) absolute abundance showed no significant differences between laboratories (Wilcoxon paired Test, p=011, n=13). For both laboratories, mean Na/K-ATPase abundances in Caco-2 were higher than for human intestines. There was no correlation in sample abundance between laboratories (p=0.43).

**P-gp** absolute abundance was significantly higher when determined in UoM versus BPh (Wilcoxon paired test, p = 0.0002, n=12). Furthermore, a moderate correlation (p=0.04, $R^2$=0.32) was found between samples. For the distal ileum, P-gp abundance was below limit of quantification (<0.125 fmol/µg) in BPh determinations.

**BCRP** absolute abundance showed no significant differences between laboratories (Wilcoxon paired test, p=0.68, n=13) and there was no correlation (p=0.27) in sample abundance between laboratories. In both laboratories higher mean BCRP abundances were observed in human samples compared to Caco-2, opposite to that seen for P-gp.

**Figure 1. A-C** – Absolute protein abundances for Na/K-ATPase, P-gp and BCRP determined by BPh (black bars) and UoM (white bars) in Caco-2 cells and human intestines. BLQ = below limit of quantification, * p = <0.05. Text above bars indicates Caco-2 growth age, total or plasma membrane (TM/PM) and HP is high passage Caco-2. **D-F –** Plots showing correlations of Na/K-ATPase, P-gp and BCRP absolute abundances between BPh and UoM. Diamonds denote human (white) and Caco-2 cells (black).

**Discussion & Conclusion:**

- Overall, UoM quantification for P-gp was significantly higher and correlated with BPh. Peptide selection, digestion or LC-MS/MS conditions may lead to differences in quantitation.
- No mean abundance or sample correlation differences exist for Na/K-ATPase or BCRP between laboratories.
- Identifying & accounting for methodological bias is crucial when incorporating data into PBPK models. However, the relevance of any differences in abundance quantification on the success of model outcomes requires ascertaining. Characterising protein losses during preparation is advocated[3] with further across-laboratory studies on matched samples required.

**References:**

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2. Vildhede *et al.*, 2014, DMD, 42, 1210-18.
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6. Sakamoto *et al.*, 2011, J Pharm Sci, 100, 4037-43