Quantitative Proteomic Map of Enzymes and Transporters in the Human Kidney: Stepping Closer to Mechanistic Kidney Models to Define Local Kinetics

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Quantitative Proteomic Map of Enzymes and Transporters in the Human Kidney: Stepping Closer to Mechanistic Kidney Models to Define Local Kinetics

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Abstract

The applications of translational modelling of local drug concentrations in various organs had a sharp increase over the last decade. These are part of the 'Model-Informed Drug Development' (MIDD) initiative, adopted by the pharmaceutical industry and promoted by drug regulatory agencies. With respect to the kidney, the models serve as a bridge for understanding animal versus human observations related to renal drug disposition and any consequential adverse effects. However, quantitative data on key drug-metabolizing enzymes and transporters relevant for predicting renal drug disposition are limited. Using targeted and global quantitative proteomics, we determined the abundance of multiple enzymes and transporters in 20 human kidney cortex samples.

Nine enzymes and twenty-two transporters were quantified (eight for the first time in kidney). In addition, >4000 proteins were identified and used to form an open database. CYP2B6, CYP3A5 and CYP4F2 showed comparable, but generally low expression, whereas UGT1A9 and UGT2B7 levels were the highest. Significant correlation between abundance and activity (measured by mycophenolic acid clearance) was observed for UGT1A9 (Rs=0.65, p=0.004) and UGT2B7 (Rs=0.70, p=0.023). Expression of P-gp ≈ MATE-1 and OATP4C1 transporters were high. Strong inter-correlations were observed between several transporters (P-gp/MRP4, MRP2/OAT3 and OAT3/OAT4); no correlation in expression was apparent for functionally related transporters (OCT2/MATEs). This study extends our knowledge of pharmacologically-relevant proteins in the kidney cortex, with implications on more prudent use of mechanistic kidney models under the general framework of quantitative systems pharmacology and toxicology.

Introduction

In addition to a central role in homeostasis and metabolic waste elimination, the kidneys represent primary organs for excretion of drugs.[1] Furthermore, there is increasing evidence of metabolic drug elimination via the kidneys, particularly by glucuronidation.[2,3] Following successes in using quantitative translation to predict *in vivo* hepatic metabolic clearance from *in vitro* data, recent progress has been made in applying this approach for predicting renal metabolic and excretion clearance within a physiologically-based pharmacokinetic (PBPK) modelling framework.[2,4] All these efforts have now moved beyond academic curiosity as the concept of 'Model-Informed Drug Development' (MIDD) has been adopted by the pharmaceutical industry to accelerate the processes involved in bringing safe and effective drugs onto the market[5,6] and recognised as a valid path by several regulatory agencies.[7,8] Nonetheless, despite advances in PBPK modelling and the translational advantages that it offers,[9–11] it is evident that information gaps in system parameters exist, particularly for the organs which were not in the front line of the initial wave of PBPK modelling. In contrast to the liver or intestine, it is not surprising to see only few attempts in quantitative assessment of drug handling proteins in the kidneys.[12–16]

PBPK modelling currently has the largest regulatory impact in the area of prediction of drug-drug interactions.[10] However, there is an increasing interest to expand and use this approach to support other MIDD initiatives, particularly in specific sub-groups of patient populations that are inadequately addressed by clinical trials.[17,18] These include paediatric and elderly patients and those with organ impairment (e.g., chronic kidney disease).[11,19,20] Extrapolation into specific sub-groups of patient populations is challenging as the information on some of the systems parameters and their interindividual variability may not be available. This is particularly evident in the case of drug transporters.[20,21] Analogously,

quantitative systems pharmacology models can also make use of quantitative 'omics' data generated using relevant systems.[22]

Protein abundance of drug-metabolizing enzymes (DMEs) and transporters is an example of important quantitative system (physiological) data required to inform robust PBPK model development and extrapolation.[11,23] In recent years, quantitative proteomic research has witnessed significant expansion and development of new methodologies, together with refinement of specific technical aspects of existing approaches.[24] At the same time, the proteomics community is actively engaged in collaborative efforts to establish principles of best-practice when generating and reporting proteomics data.[25] Such efforts, which include inter-method and inter-laboratory comparisons,[24,26] increase the level of confidence in the data used for meta-analyses and inter-protein correlations that subsequently inform PBPK modelling.[27]

Despite all the progress in supporting more mechanistic kidney models, there are only a few studies that quantified DMEs and transporters in the human kidney, while functional activity data are generally lacking.[12,14–16,28,29] Hence, the aim of the current study was to quantify protein abundance of key DMEs, namely cytochrome P450 (CYP) and uridine-5′-diphospho-glucuronosyltransferase (UGT) enzymes, together with drug transporters in kidney cortex from 20 human nephrectomy donors using targeted-QconCAT proteomics. In addition, global proteomics was performed to quantify many more transporters over the targeted approach, assist description of the protein composition of the same samples and allow inter-method comparison. Mycophenolic acid glucuronidation clearance was determined in each individual sample for verification of the UGT abundance data and as evidence of functional activity. Inter-correlations between individual expression of 31 kidney cortex CYPs, UGTs and transporters, together with cross-family comparisons, were explored.

Methods

Human kidney samples

Human kidney cortex samples were obtained from patients undergoing nephrectomy (n=20) from the section contralateral to the tumour site and considered " histologically normal" by pathologist. Kidney cortex pieces were snap frozen within 1h of excision and stored at 80°C, as detailed previously.[2] The samples were provided by Central Manchester University Hospitals NHS Foundation Trust (CMFT) Biobank, Manchester, UK, with research ethical approval (13/LO/1896) from the National Research Ethics Service (NRES) Committee, London, UK.

Demographic and corresponding UGT polymorphism data, together with details of preparation of microsomal fractions from the kidney cortex samples were reported previously[2] and have been summarised in Supplementary Table S1. Microsomal protein losses were corrected using glucose-6-phosphatatse as a marker.[2]

Preparation of kidney microsomal samples for proteomic analysis

The human kidney microsomal (HKM) protein was digested using an optimized filter-aided sample preparation (FASP) protocol.[30,31] FASP was selected over in-solution digestion protocol for its simplicity, time-efficiency, efficient removal of contaminants, and its applicability to clinically-relevant enzymes and transporters.[24,32] Details of digestion of kidney proteins and nano LC-MS/MS analysis are in Supplementary Information.

Accurate mass and retention time (AMRT) method was performed as a targeted proteomic approach to quantify enzymes, transporters, a tight junction protein and a plasma membrane marker within human kidney cortex samples. The untargeted quantification was performed using QconCAT-based abundance of MDR1 as a reference. The abundance of each relevant protein was quantified by the Hi-N method, as described previously.[33] AMRT

and untargeted data analysis were performed using MaxQuant version 1.5.5.1 (Max Planck Institute of Biochemistry, Martinsried, Germany).

Proteins were identified by searching against a reference human proteome database containing 71,599 entries (UniProt, May 2017) and a decoy database of the predicted proteome for *Homo sapiens*. Percentage identical peptides (PIP) and percentage identical proteins (PIPr) were estimated as previously described [31,33] to assess the integrity of replicate analyses (Figure S3) and the overall similarity of samples (Table S2).

Protein subcellular localization and functional analysis

To facilitate the comprehensive characterisation of HKMs, the sub-cellular localization of all identified proteins (*n*=3946) was annotated according to three databases: Gene Ontology (GO, http://geneontology.org/), UniProtKB (https://www.uniprot.org/) and the Human Protein Atlas (HPA, https://www.proteinatlas.org/), by searching the repositories for the gene names. In addition, the PANTHER (Protein ANalysis THrough Evolutionary Relationships) classification system version 14.0 (http://www.pantherdb.org/) was used to assign protein functional class. Classes related to metabolism and functional plasma membrane proteins were annotated specifically. Identified proteins were those observed with at least one unique peptide with a significantly high score.

Comparison of UGT expression and functional activity data

To verify the expression data obtained by the quantitative proteomics, assessment of direct correlation between the quantified abundance of UGT1A9 and UGT2B7 and their functional activity was performed using previously reported unbound mycophenolic acid glucuronidation clearance data generated in the same samples. Clearance was obtained by substrate depletion at a low drug concentration; all details of functional assay have been reported in[2].

Inter-organ comparison of enzyme and transporter expression data

Expression data for enzymes (CYP2B6, CYP3A5, CYP4F2, UGT1A1, UGT1A3, UGT1A4, UGT1A6, UGT1A9 and UGT2B7) and transporters (P-gp, MRP2, MRP6, OAT2 and MATE-1) obtained by targeted proteomics (with the exception of MRP2 and MRP6 that were measured by the label-free Hi-N method) in kidney cortex (n=20) were compared to the previously reported expression data for the same proteins in the histologically normal intestine (n=16) and healthy liver (n=24) obtained by the targeted methodology.[34–36]

Statistical data analysis

All statistical analysis was performed using Microsoft Excel 2010, GraphPad Prism version 7.04/7.0c for Windows (GraphPad Software, San Diego, California, USA). Nonparametric statistics were used because a considerable proportion of the dataset did not follow normal distribution. Normality of data distribution was assessed using three tests: D'Agostino−Pearson, Shapiro−Wilk, and Kolmogorov−Sminov normality tests. The Spearman rank-order correlation (Rs) test, with *t*-distribution of the *p*-values, was used to assess enzyme abundance−activity correlation and inter-correlation between protein abundance levels. The level of scatter of data was evaluated by linear regression (R^2). The relationship between transporter-transporter, enzyme-enzyme and enzyme-transporter levels and expression covariates, sex and age, were assessed using Mann-Whitney *U*-test. The abundance data were described as mean±SD, coefficients of variation (CV). For inter-correlations, Rs > 0.60 and R^2 > 0.15 and a Bonferroni-corrected p-value of < 0.01 were considered for significant correlations taking into account the effect of the units of measurement.

Results

In this study, we quantified expression of key metabolic enzymes (CYPs and UGTs) and transporters in 20 human kidney cortex samples. Evaluation of two quantitative proteomic methods, targeted and global, was performed. Inter-correlation in protein

expression was assessed between all 31 enzyme/transporter proteins quantified. Finally, comparison of the expression data between liver, intestine and kidney was performed for selected proteins where data were available in all three tissues.

Assessment of protein abundance measurements

QconCAT and label-free approaches were used for the analysis of fresh frozen kidney microsomes. For the AMRT approach, the MetCAT[36] standard was used to measure the abundance of clinically relevant DMEs from the CYP450 family (CYP2B6, CYP3A5 and CYP4F2) and UGT family (UGT1A1, UGT1A3, UGT1A4, UGT1A6, UGT1A9 and UGT2B7). The majority of proteins were quantified in all 20 cortex samples, with the exceptions of UGT1A3 and UGT1A4, which were measurable in only eight and seven samples, respectively. In the same experiment, the TransCAT[36] was used to measure protein abundance of a membrane marker (Na^+/K^+-ATPase) and relevant drug transporters, namely P-gp, MRP4, OCT3, OAT2, MATE-1, OATP4C1, OST-α, OST-β, and CDH-23; the latter is a calcium-dependent cell adhesion proteins.

Abundance of CYP and UGT enzymes in human kidney samples using the targeted approach

In total, abundance of 9 CYP and UGT enzymes was measured in kidney cortex samples. To our knowledge, this is the first report of CYP2B6, CYP3A5, CYP4F2, UGT1A1, UGT1A3 and UGT1A4 abundance quantification in the human kidney. The median of the three CYP enzymes was comparable (0.20, 0.15 and 0.17 pmol/mg of protein for CYP2B6, CYP3A5 and CYP 4F2, respectively; Table 1), with CYP4F2 data showing the largest inter-individual variability (CV>50%, Figure 1A). More than 100-fold difference in expression was noted in the quantified UGTs in the kidney cortex and the abundance followed the rank order: UGT1A9 > UGT2B7 > UGT1A6 > UGT1A4 > UGT1A3 > UGT1A1 (Figure 1B). Analogous to CYPs, some of the UGTs showed low expression (e.g., UGT1A1, 1A3, 1A4), approximately 18-fold lower than UGT1A9 (the most abundant DME in the kidney cortex).

Expression data (pmol/mg microsomal protein) per individual donor (n=20) for all proteins quantified in this study are presented in Table S3. In addition to the current study, literature reported expression data for enzymes and transporters in the kidney were collated (Table S4). Data from the current study were in general agreement in relation to the rank order of abundance with existing literature data for enzymes where such data were reported (UGT1A9 > UGT2B7 > UGT1A6).

Correlation between UGT protein expression and functional activity

The correlation between UGT1A9 and UGT2B7 abundance, quantified by the AMRT approach, and functional activity of these enzymes, assessed by mycophenolic acid glucuronidation was evaluated. The analysis demonstrated strong, significant, and positive correlation between abundance and enzyme activity for these two UGTs (Rs > 0.60, p< 0.023) and demonstrated moderate scatter ($R^2 \geq 0.16$, Figure 2). As mycophenolic acid is not a specific substrate for either of these enzymes, a perfect abundance-activity correlation was not expected for either individual enzyme, unless abundances themselves were correlated, which was not the case for UGT1A9 and UGT2B7 (see below).

Correlations of protein expression between renal CYP and UGT enzymes

Expression of 9 CYP and UGT enzymes were compared to investigate any potential enzyme inter-correlations (e.g., expression of CYP3A5 vs. UGT1A9). The analysis showed that no significant correlation could be established between the enzymes investigated (Rs<0.30, p>0.40).

Protein abundance of ABC and SLC transporters in human kidney samples using targeted approach

In total, eight transporters were measured in kidney cortex samples, in addition to one adhesion protein (cadherin-23) and one plasma membrane marker (Na^+/K^+-ATPase). Out of ABC transporters, median expression of P-gp was 6-fold greater than MRP4. P-gp and MATE-1 were the most abundant proteins (median values; 8.36 and 6.26 pmol/mg of protein, respectively) followed by OATP4C1, OAT2, OST-β, OCT3 and OST-α (Figure 3, Table 1). The plasma membrane marker (Na^+/K^+-ATPase) did however show significantly higher expression in the human kidney than the other transporters.

Global analysis of identified human kidney microsomal proteins

In addition to targeted proteomics, global proteomic analysis of human cortex samples was performed. To characterise kidney cortical microsomes and their proteomic make-up, proteins identified (n = 3946) were assigned to their sub-cellular location and function using the GO, UniProtKB, HPA and PANTHER databases (Figure S1). Approximately 23% and 25% of identified proteins were assigned to the plasma membrane and endoplasmic reticulum, respectively. The analysis showed that 48% of all proteins were either reticular or cell membrane proteins, whereas 20%, 18% and 14% of proteins were mapped to the cytoplasm/cytosol, mitochondria and nucleus, respectively (Figure S1A). It should be noted that various proteins can be expressed in multiple subcellular locations, which resulted in the assignments exceeding 100% of the total number of proteins. Proteins were also assigned into several functional classes, with 72% of all identified proteins shown to belong to a defined class. Of relevance, 447 proteins were functional at the plasma membrane and 865 proteins had a metabolic role (Figure S1B). Other proteins included nucleic acid binding proteins (372), enzyme modulators (271) and signalling proteins (103).

Reproducibility of kidney global proteomics data

Similarity between identified peptides in the analytical replicates (2 for each sample) of the 20 kidney samples was evaluated. In each sample, a very high similarity was seen between the peptides identified in the analytical replicates, with PIP varying between 63-73% (Table S2). PIP values in this range indicate a high degree of consistency in both sample preparation and instrument performance and are reassuring, especially in view of the long mass spectrometry run required by 20 samples in duplicate. In addition, similarity between the replicates of the 20 cortex samples were evaluated by proteome profiles and reproducibility of both peptide and proteins (PIP and PIPr).[32,33] PIP varied between 26% and 65%, and PIPr varied between 57% and 83% (Figure S3). As the LC-MS methodology applied here has been used extensively by our group,[30,32,33] the variations noted are likely to reflect true biological differences in the kidney cortex samples rather than variability in preparation or analysis.

Transporters identified and quantified using the global approach

This is the first global proteomic study of the human kidney cortex and it allowed identification of 4635 proteins from an average of 39,667 peptide matches per nanoLC-MS/MS run. After removal of duplicated and truncated proteins, this number decreased to 3946. This step was to ensure that only functional proteins with confirmed identification were used in subsequent analysis.

Out of the 14 transporters (MRP2, MRP3, MRP6, OCT2, OCTN1, OCTN2, OAT1, OAT3, OAT4, OATL4, OCTL3, SGLT1, SGLT2 and BTR1) identified and quantified using the global approach, 5 proteins (MRP6, OATL4, OCTL1, BTR1, SGLT1) were quantified for the first time. The median value for BTR1 was the highest, followed by SGLT2 (17.2 pmol/mg protein, n=15, for BTR1 and 14.1 pmol/mg protein, n=17, for SGLT2), whereas the

median expression of MRP2 was the lowest (0.60 pmol/mg protein, n=7) (Table S5, Figure 4).

The protein abundance of the transporters quantified showed very high variability with a CV of 40%-139% except for OAT4, which was the most variable transporter (CV=145%) (Figure 4). Both transporters and UGT enzymes quantified showed similar variability with a CV of 40%–139% for transporters and 10%-136% for UGT. The analysis showed no correlation between expression of transporters/enzymes and age (43-83 years) or sex (n=16 men and n=4 women) (unpaired t-test, $p>0.05$). Direct comparison of all transporters obtained by the global proteomics to targeted method was not feasible due to lack of quantification of some proteins by the targeted approach. However, where available, comparison of protein abundances estimated by the label-free and targeted approaches showed good agreement between the methods.

The absolute protein abundance for these 14 transporters in the samples was calculated by normalizing the total signal to P-gp; the abundance of this protein (P-gp) has been determined by QconCAT-based targeted analysis. Table S4 lists average abundance transporters with relevant references to other complementary data from targeted studies. To validate the label-free results, P-gp protein expression in kidney microsomes was compared between targeted and label-free approaches. High correlation in P-gp protein abundance was observed between the two proteomic methods (Rs=0.80, $p<0.001$, $R^2=0.72$) (Figure S2).

Correlations of protein expression between renal transporters and enzymes

Potential inter-correlations between expression of transporters in the human kidney cortex were investigated, as summarised in Table 2. Strong inter-correlations between P-gp and other renal transporters were observed, namely MRP4 (Rs= 0.93, $P<0.0001$, $R^2=0.71$), OAT1 (Rs = 0.84, $p<0.0001$, $R^2=0.50$) and OATP4C1 (Rs=0.78, $p=0.003$, $R^2= 0.66$). MRP4 showed strong correlations with SGLT1 (Rs=0.91, $p<0.0001$, $R^2=0.56$) and with OATP4C1

(Rs=0.96, p=0.003, R^2=0.86). In addition, OAT1 was correlated with OATP4C1 (Rs=0.95, p<0.0001, R^2=0.80) and with OCT2 (Rs=0.83, p<0.0001, R^2=0.96). OAT4/SGLT2 (Rs=0.93, p=0.001; R^2=0.95) were also correlated. The analysis of inter-correlations between the expression of individual enzymes (either CYPs or UGTs) and transporters showed no clear cross-family correlations (Rs generally <0.30).

Comparison of enzyme and transporter protein abundance in kidney, liver and intestine

In the current analysis, proteomic data on CYPs, UGTs and transporters in kidney cortex were compared with previous studies that quantified the abundance of the same proteins in the histologically normal intestine and healthy liver using the same quantification method and proteomic workflows.[30,34–36] Only the proteins for which the data were available from all the three tissues were included in this analysis.

Generally, the expression of kidney UGTs (UGT1A1, 1A3 and1A4) was up to 200-fold lower than those in liver (Figure 5); the smallest difference was seen for UGT1A6, 2B7 and 1A9 (63-, 51- and 14-fold). Comparison of CYP2B6, 3A5 and 4F2 expression showed the highest fold difference for CYP2B6 (200-fold), followed by 3A5 (100-fold) and 4F2 (54-fold) (Figure 5); in all the cases, higher CYP expression in the liver was consistent with the trend seen with UGTs. In addition, comparing expression data between kidney and intestine, UGT1A1, UGT1A3 and UGT1A6 showed higher expression in the intestine (the most pronounced difference was 10-fold for UGT1A1), whereas there was no difference in the UGT2B7 expression between the intestine and kidney.

In contrast to enzymes, transporters such as P-gp and MATE-1 were present at very high levels in the kidney relative to liver, whereas expression of MRP2, MRP6 and OAT2 was comparable. The expression of P-gp in the kidney and intestine were 16-fold and 2-fold higher relative to the liver. MRP2 and MRP6 expression in kidney was comparable to the liver; analogous comparison to the intestinal data indicated that expression of MRP2 was on

average up to 4-fold lower in the kidney. The average expression value of OAT2 was approximately 2.3-fold higher in the liver (0.79 and 1.84 pmol/mg protein for the liver and kidney, respectively). The MATE-1 abundance in liver was approximately 30-fold lower in the liver relative to the kidneys (Figure 5).

Discussion

The kidneys govern excretion of drugs/metabolites and therefore play a crucial role in regulating systemic and local tissue exposure. The current study applied targeted and global LC-MS/MS-based proteomics to quantify key CYPs, UGTs and transporters in 20 kidney cortex samples. In addition, label-free global proteomic characterisation was performed for the first time and identified >4000 proteins in human kidney.

For several proteins investigated here, no expression data have previously been reported in the human kidney cortex, including six enzymes (CYP2B6, CYP3A5, CYP4F2, UGT1A1, UGT1A3, and UGT1A4) and eight transporters (MRP6, OATL4, OCTL3, OCTN2, SGLT2, BTR1, OST-α, OST-β). No correlation could be established in expression of CYPs, UGTs and transporters in kidney with age; this trend was not surprising considering that donor population in the current analysis was adult, whereas more pronounced ontogenic differences are expected in paediatric populations.[37] In addition, there was no significant relationship between protein expression and sex of donors. Both of these observations are consistent with similar analysis reported for the liver, intestine and brain.[30,33,38] Only CYP2B6, CYP3A5 and CYP4F2 were quantified in the renal cortex; all these enzymes showed comparable expression. However, CYP4F2 showed the highest inter-individual variation (51%) in contrast to CYP2B6 (10%). Low CYP expression in the kidneys is consistent with previous reports,[23] indicating minor contributing role of renal oxidation to drug elimination.

There is a paucity of reports on UGT quantitative proteomics in human kidney (summary of available data in Table S4), likely because these enzymes are membrane-embedded and show very high sequence homology. UGT1A9 had the highest abundance in the current samples, followed by UGT2B7 and UGT1A6, in agreement with previous data.[28,29] Noticeably, high inter-individual variability (CV%) was observed in UGT1A6

(108%), UGT1A9 (95%) and UGT2B7 (136%) expression, in agreement with a previous study using targeted proteomics.[28] Low expression of UGT1A9 and UGT2B7 was noted in individual donors who had polymorphisms of these enzymes; however, our current dataset was not sufficiently large to establish any clear relationship between UGT1A9/2B7 genetic polymorphism and their expression (data not shown). Inter-individual variability in UGT expression in kidney cortex was notably higher than variability in CYP expression in the same individuals (10-51%).

There are particular challenges in measuring abundances of human renal transporters as their expression and localisation can change in different diseases.[39] As diseased samples are generally the only ones available for proteomic analysis, obtaining histologically normal tissue from such donors is very important. Eight transporters were quantified here using targeted proteomics in following rank order (from highest to lowest): MATE-1≈P-gp, OATP4C1, MRP4, OCT3, OST-α, OST-β, OAT2 (Figure 3). OATP4C1, OST-α and OST-β were measured for the first time in this study. The rank order and the expression levels of certain transporters (P-gp, OCT3) differed from existing literature. This is not unexpected, considering donor differences and that some previous reports do not specify the kidney region of origin.[12,14–16] In addition, it is important to appreciate that existing kidney proteomic data are generally based on small cohorts, which makes it challenging to fully appreciate biological variability in the expression of some of these proteins. For instance, certain studies report quantitative transporter data from only 4-5 donors (Table S4). Our MRP4, OAT2 and MATE-1 expression data were similar to those reported previously for the same kidney region.[12]

In addition to transporter proteins, the current study measured cadherin-23, which is expressed in various tissues, including the kidneys. Previous studies identified a strong association of cadherin-23 with estimated glomerular filtration rate, suggesting a critical role

in normal kidney function.[40] In the current study, the expression of cadherin-23 was comparable to OCT3 expression. As different pathophysiological changes can affect its expression in the kidney, measurement of this protein can be a potential proteomic marker of disease progression. Expression of Na+/K+-ATPase was the highest of all the quantified transporter proteins, consistent with previous studies,[12,15,16] confirming its important role in maintaining ionic gradients and regulating pH across plasma membrane. Indirectly, measurements of Na+/K+-ATPase also confirm the robustness of the LC-MS/MS proteomic methods applied here.

This is the first global proteomic study aimed at analysing human CYPs, UGTs, and transporters in human kidney cortex. Out of 4000 proteins that were identified, we focused on 14 proteins relevant to renal drug elimination. However, it is important to acknowledge the possibilities of using the global approach beyond proteins involved in traditional pharmacokinetics, in particular for quantification of protein expression in different disease states. Global proteomic method identified other physiologically relevant transporters (e.g., fatty acid transporter, FATP2). This transporter is expressed on the apical proximal tubule membrane and is associated with regulation of lipoapoptosis, with potential link with chronic kidney disease.[41] In addition, the current analysis identified transporters from the ABCD family (1, 3), which are retained in an open database to explore.[42] Abundance of five transporters (MRP6, OATL4, OCTL1, SGLT1, and BTR1) are reported for the first time in human kidney (Figure 4). In addition to renal drug elimination, these transporters are linked with several diseases, e.g., MRP6 suppression leads to arterial calcification in chronic kidney disease patients,[43] while downregulation of the urate transporter OATL4 can cause renal hypouricaemia.[44] The ability of label-free proteomics to quantify novel targets, such as BTR1 (genetic polymorphism associated with congenital hereditary endothelial dystrophy),[45] demonstrates potential applications in proteomic characterization of different diseases with

tissues obtained from such patient populations. This is of particular relevance for supporting development of PBPK models to inform dosage regimen design for complex patient cohorts with multiple existing comorbidities.

Comprehensive evaluation of inter-correlations of protein expression and its consideration in the PBPK models is essential for development of plausible virtual populations and simulation of realistic inter-individual variability in pharmacokinetics.[46] As such, increasing number of studies are exploring inter-correlations in protein expression.[30,33,36] In our previous studies involving human liver and intestine, strong correlation was established between individual CYPs, UGTs and across families (CYP2C9 and UGT2B7).[30,34,36]

Strong inter-correlations reported for hepatic MRP2/MRP3[47] and intestinal CYP3A4/P-gp[36] are not surprising considering common protein regulation, but in other cases, e.g. renal MRP4/OATP4C1, the physiological relevance is not fully established. This study also explored correlation across protein families (e.g. UGT- transporter), particularly the ones that are connected functionally in renal elimination (e.g. OCT2 and MATEs) or MRP2 transport of glucuronide metabolites formed by UGTs.[48] However, no strong inter-correlation in the expression of these protein pairs could be established in kidney cortex, in contrast to very strong inter-correlations observed for other proteins (MRP6/OAT3, MRP2/OAT3, P-gp/OAT1 and OAT3/OST-α). These correlations are novel, with only one of the uncovered correlations (OAT4/SGLT1) previously reported to be moderate.[12]

The cross-organ comparison of in-house expression data of CYPs, UGTs and transporters in the kidney, intestine and liver showed 50–200 times lower CYP abundance in the kidney than in the liver. As our kidney work focused only on CYP3A5, CYP2B6 and CYP4F2, comparison with the intestine was not possible, as these enzymes were not quantifiable in intestine. The UGT abundance in the kidney was generally very low (mean

0.14-2.62 pmol/mg protein) compared to liver, whereas the expression of UGTs in the intestine and kidney were comparable (within 3-10 fold), especially for UGT2B7. The expression of transporters in the kidney was overall higher than in the liver, whereas trends were variable between intestine and kidney.

In-depth analysis of global proteomic data identified 14 additional transporters compared with targeted proteomics. This highlighted the complementary nature of parallel use of proteomic methods, in addition to cross-method verification of overlapping data. The 'open access' global proteomic data represent a valuable resource for investigation of proteins with relevance to certain disease phenotypes. These can support translation of the impact on drug-related metabolic/transporter pathways on local concentrations in various kidney regions as well as pharmacological/toxicological consequences.

This study is a step that takes MIDD closer to more prudent mechanistic kidney models. Such models were introduced with specific objective on translation of transporter data obtained from *in vitro* studies.[49] Usage of such models might be accelerated further by recent successes in modelling of endogenous biomarkers of renal excretion in patient populations,[20] as well as applications in complex interplay of multiple factors and permutations of various scenarios in relation to renal drug disposition.[4] However, these models require considerable expansion of existing knowledge of human kidney protein abundance, some of which is reported here for the first time.

Study Highlights

WHAT IS THE CURRENT KNOWLEDGE ON THE TOPIC?

The kidney expresses a variety of proteins playing major roles in renal elimination. Some of these enzymes and transporters have been quantified in a limited number of cases, but, for many key proteins, there is a complete lack of quantitative data.

WHAT QUESTION DID THIS STUDY ADDRESS?

Expression of multiple enzymes and transporters was quantified in human kidney cortex using targeted and global proteomics. The study provides the most robust understanding of inter-correlations in expression of renal enzymes and transporters, cross organ comparison to liver and intestine and establishes relationship to functional glucuronidation data .

WHAT DOES THIS STUDY ADD TO OUR KNOWLEDGE?

The unique dataset generated here (> 4,000 proteins) includes quantitative measurements of proteins in kidney cortex for the first time. Protein expression reported for nine enzymes and twenty-two transporters relevant for the metabolism and transport of drugs in the human kidney cortex.

HOW MIGHT THIS CHANGE CLINICAL PHARMACOLOGY OR TRANSLATIONAL SCIENCE?

The data presented here are an essential resource for systems biology models to facilitate quantitative translation of renal drug disposition. The study provides a protein quantification framework that can be applied to disease populations and therefore support refinement of PBPK kidney models in specific populations.

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Author contributions

Z.M.A.-M., B.A., A.G and A.R.-H. wrote the manuscript; Z.M.A.-M., D.S., B.A., J.B , A.G., and A.R.-H. designed the research; Z.M.A.-M., D.S., and B.A. performed the research; Z.M.A.-M., B.A and J.B. analyzed the data; Z.M.A.-M. contributed new reagents/analytical tools.

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Figure 1: Expression data for cytochrome P450 (A) and UGT (B) enzymes in 20 human kidney cortex samples. In the box and whisker plots, the lines indicate the median values, the whiskers indicate the minimum and maximum abundance values, and + represents mean values. The number of donors where enzyme abundance were measured is shown above each represented dataset.

Figure 2: Correlation of the abundance of uridine 5′-diphospho-glucuronosyltransferase (UGT) enzymes measured using targeted AMRT and enzyme activity. Mycophenolic acid intrinsic clearance ($CL_{int,u}$) data were obtained by substrate depletion method in the same individuals and reported elsewhere. Strong and significant correlations (Rs > 0.60, $P < 0.023$) with moderate scatter ($R^2 \geq 0.16$) are shown between functional data and corresponding UGT1A9 and UGT2B7 abundance. Linear regression analysis was therefore performed, resulting in slopes of 0.0036 and 0.0019 for UGT1A9 and UGT2B7, respectively ($R^2 = 0.67$).

Figure 3: Expression data for eight ABC and SLC transporters in 20 human kidney cortex samples obtained by targeted proteomics. Cadherin-23 is an adhesion protein and Na^+/K^+-ATPase is a marker protein. The box and whisker plot lines indicate the median values, the whiskers indicate the minimum and maximum abundance values, and + represents mean values. The number of donors where transporter abundance were measured is shown above each represented dataset.

Figure 4: Quantification of 14 efflux and uptake transporters in human kidney cortex (n=20) using the global method. Data points represent individual kidney cortex abundance and lines indicates median abundance of each protein. Expression levels were normalized to the MDR1 expression in each sample. Incidentally, one donor showed very high expression of all transporters investigated.

Figure 5: **Comparison of fold difference in the expression of 14 cytochrome P450, UGT enzymes and transporters in the kidney and intestine relative to the liver**. Abundance data for the liver and intestine (expressed in pmol/mg of total protein) have been reported elsewhere [34–36] and were obtained by targeted proteomics.

Supplementary Information

Table S1 Demographic and corresponding UGT polymorphism data in individual human kidney samples.

Table S2 Assessment of the integrity of replicate analyses.

Table S3 Abundance of target enzymes and transporters in membrane fractions from individual kidney samples.

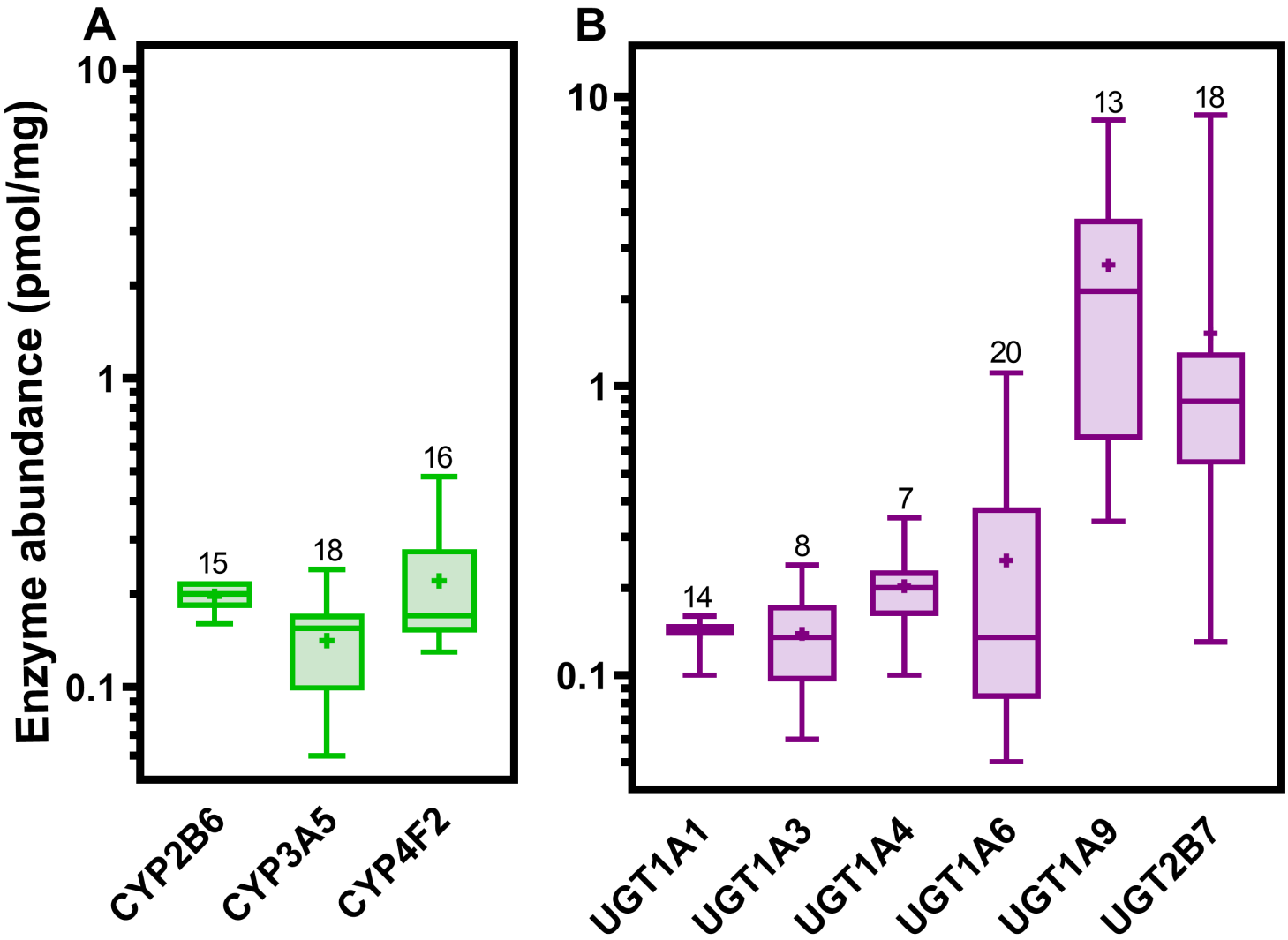
Table S4 Literature data of the investigated cytochrome P450 (CYP), uridine 5'-diphospho glucuronosyltransferase (UGT) abundance enzyme, ABC and SLC transporter abundance levels in human kidney microsomes**.**

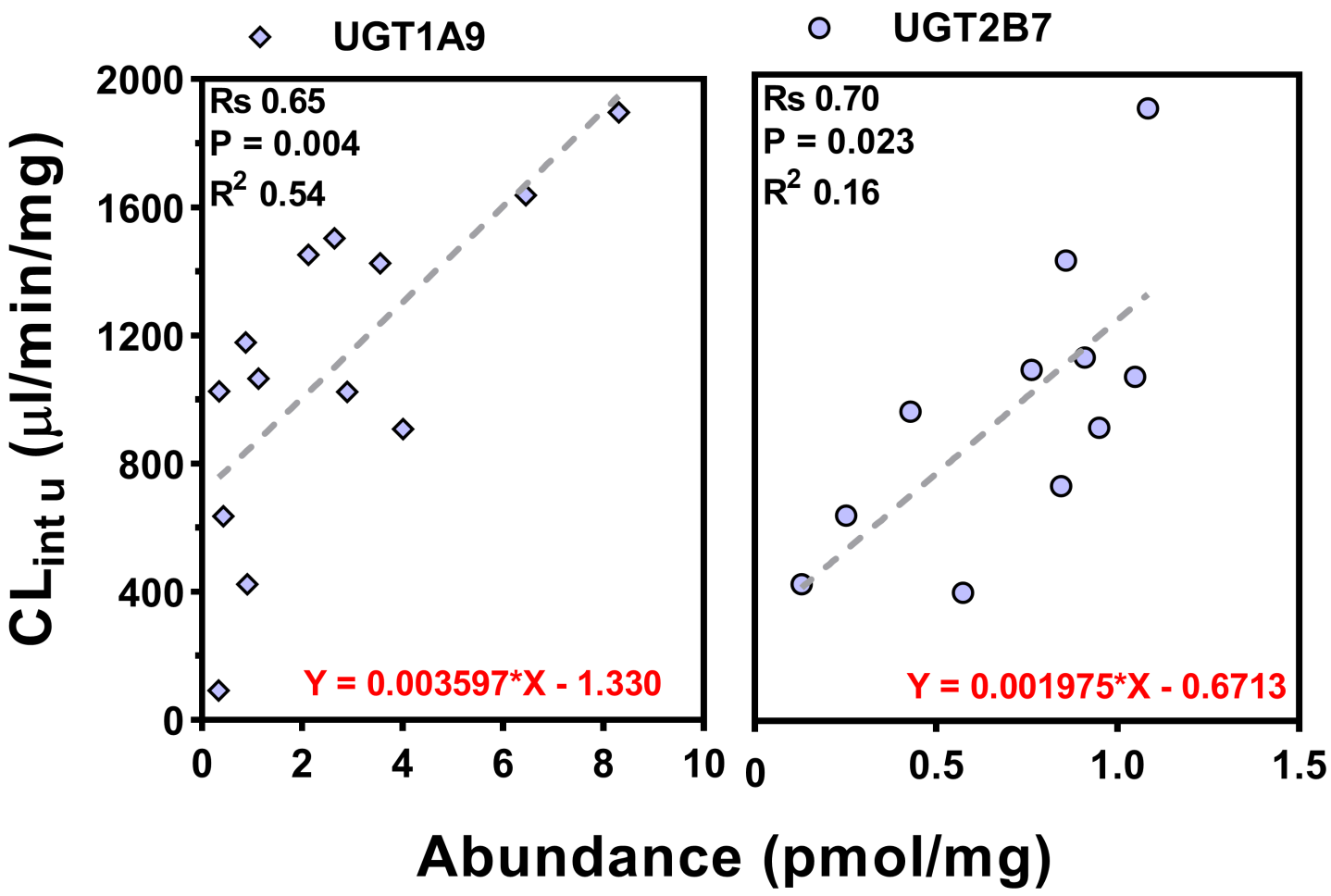
Table S5 Expression levels of quantifiable transporter (pmol/mg total protein) in the human kidney cortex using global method.

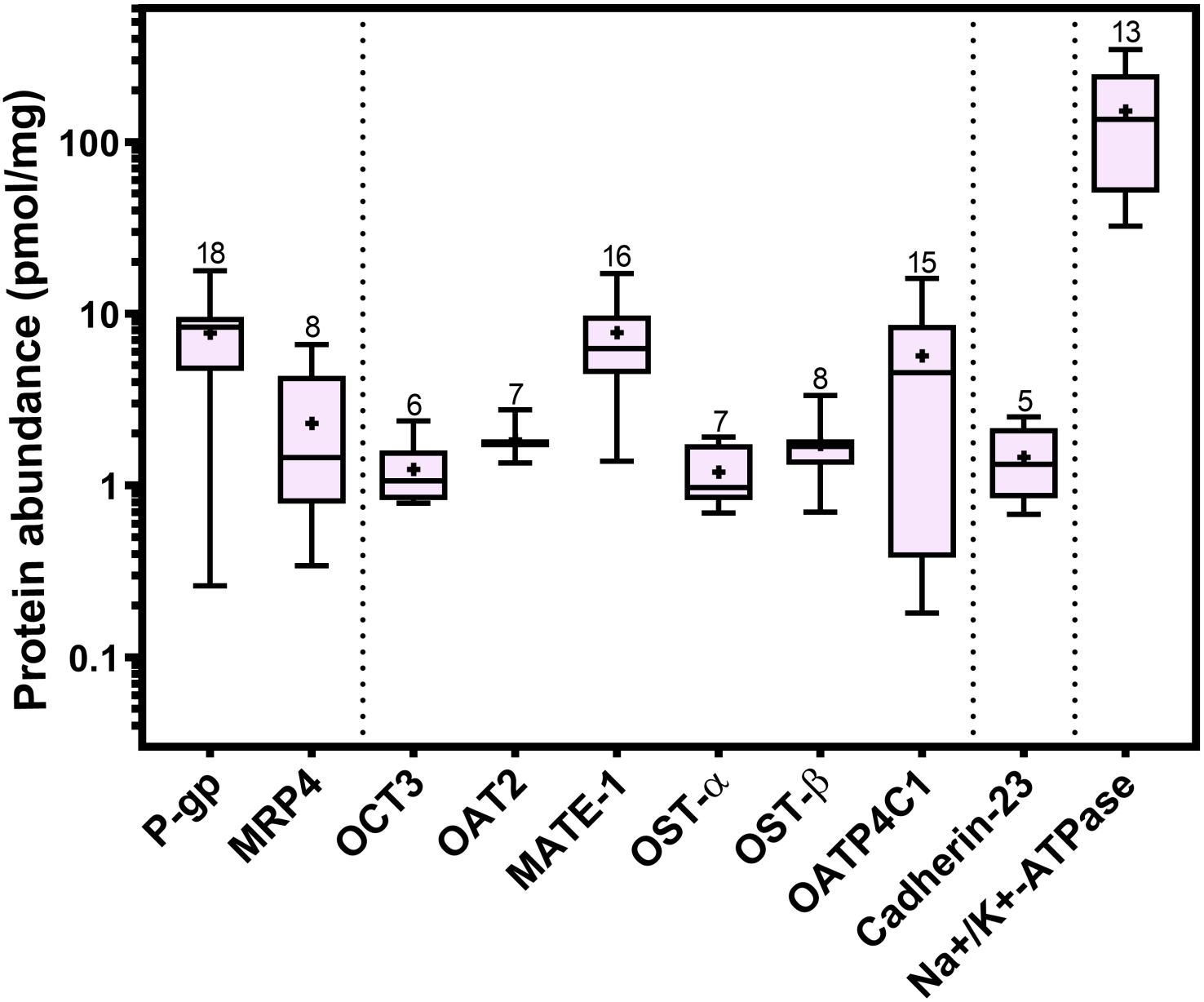
Figure S1 Localization and functional assignment of proteins identified in human kidney cortex microsomes.

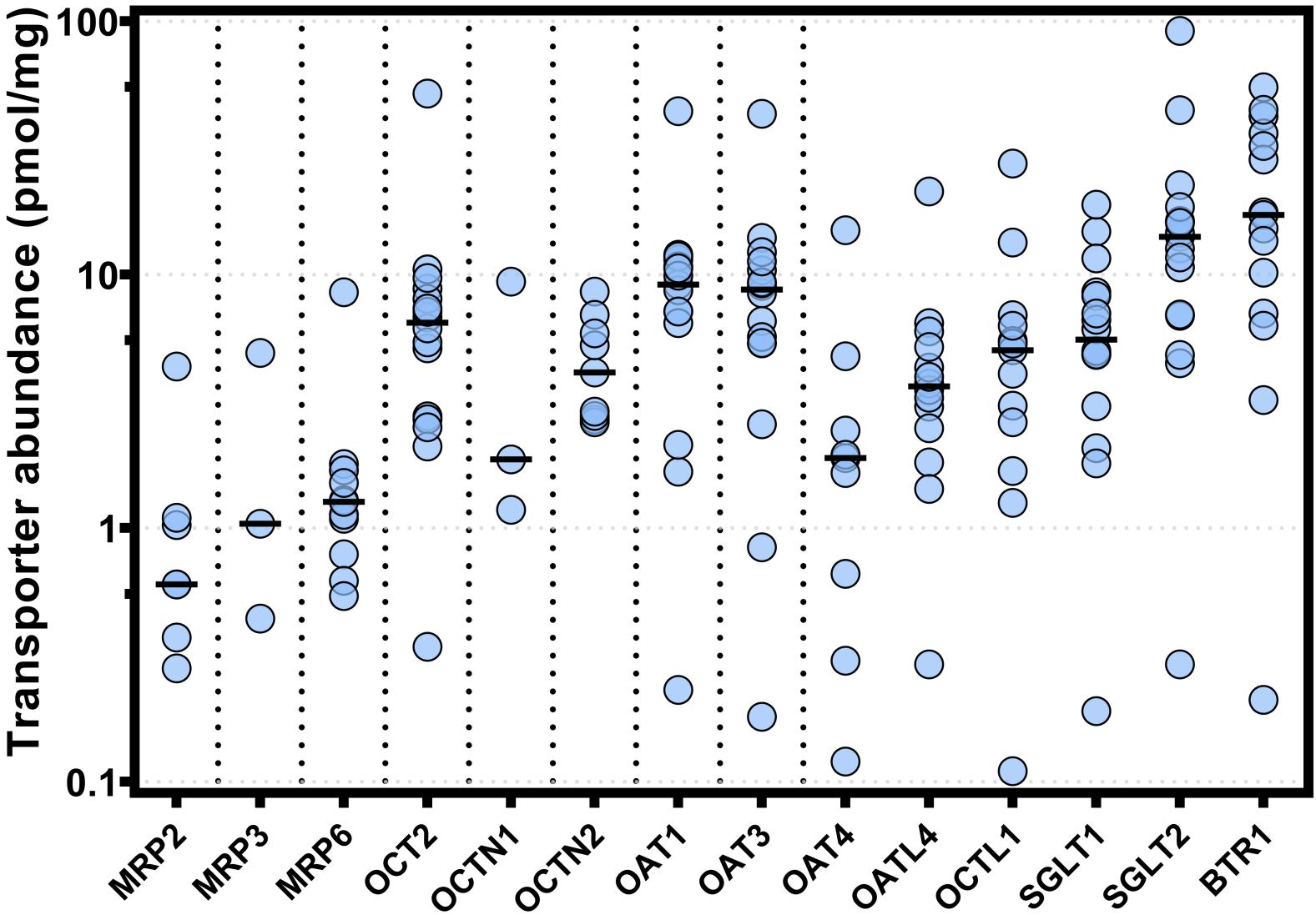
Figure S2 Correlation analysis shows agreement between P-gp individual transporter measurements by targeted AMRT and label-free approaches across 13 samples.

Figure S3 The similarity matrix between biological samples based on percentage identical peptide (PIP) and percentage identical protein (PIPr) measured using global method.


A
B
Enzyme abundance (pmol/mg)
10
1
0.1
15
18
16
14
8
7
20
13
18
CYP2B6
CYP3A5
CYP4F2
UGT1A1
UGT1A3
UGT1A4
UGT1A6
UGT1A9
UGT2B7


UGT1A9
UGT2B7
Rs 0.65
P = 0.004
R2 0.54
Rs 0.70
P = 0.023
R2 0.16
Y = 0.003597*X - 1.330
Y = 0.001975*X - 0.6713
CLint u (µl/min/mg)
Abundance (pmol/mg)
2000
1600
1200
800
400
0
0
2
4
6
8
10
0
0.5
1.0
1.5


Protein abundance (pmol/mg)
100
10
1
0.1
18
8
6
7
16
7
8
15
5
13
P-gp
MRP4
OCT3
OAT2
MATE-1
OST-α
OST-β
OATP4C1
Cadherin-23
Na+/K+-ATPase


Transporter abundance (pmol/mg)
100
10
1
0.1
MRP2
MRP3
MRP6
OCT2
OCTN1
OCTN2
OAT1
OAT3
OAT4
OATL4
OCTL1
SGLT1
SGLT2
BTR1

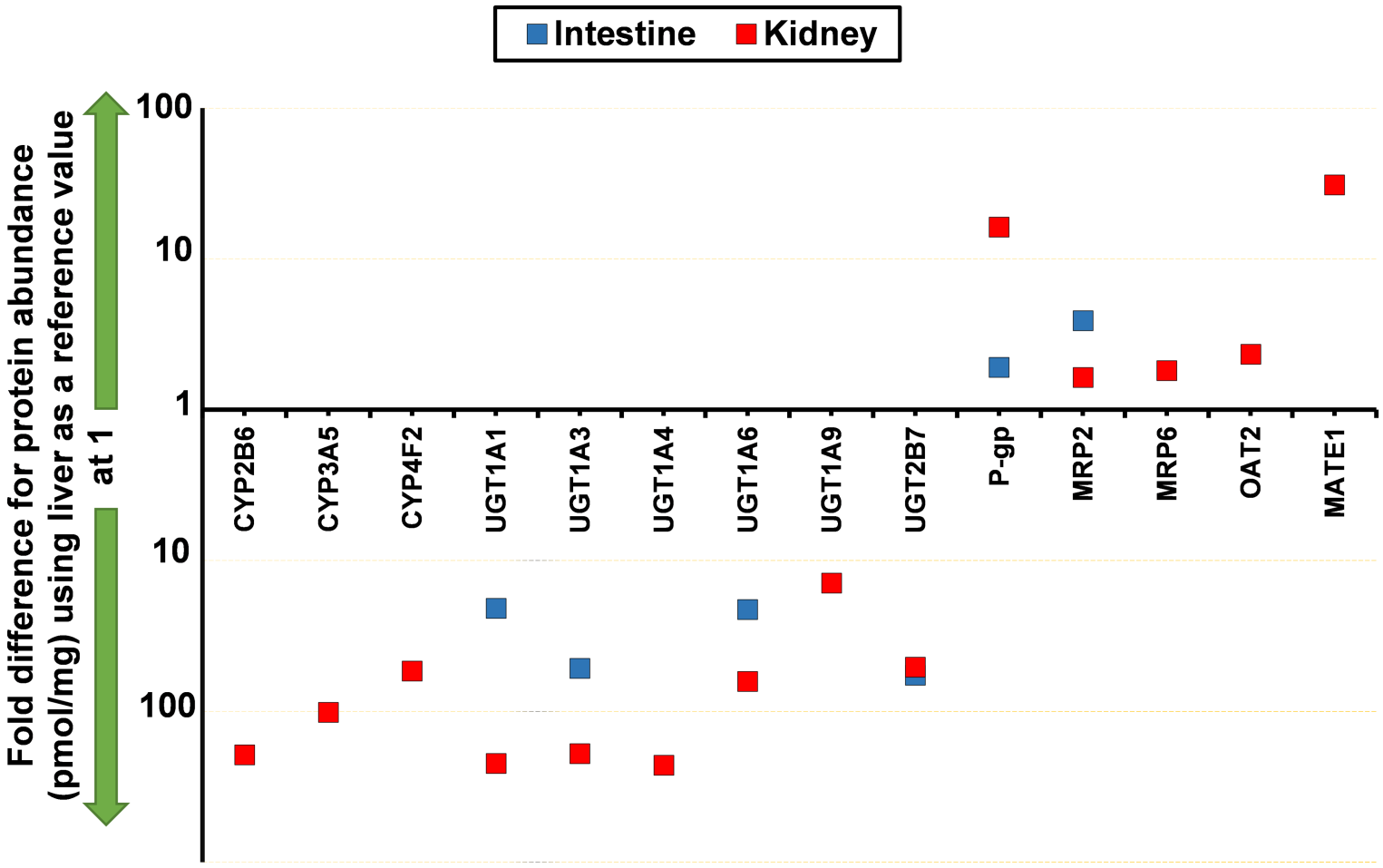

Intestine
Kidney
Fold difference for protein abundance (pmol/mg) using liver as a reference value at 1
100
10
1
10
100
CYP2B6
CYP3A5
CYP4F2
UGT1A1
UGT1A3
UGT1A4
UGT1A6
UGT1A9
UGT2B7
P-gp
MRP2
MRP6
OAT2
MATE1

Table 1. Expression levels of cytochrome P450 (CYP) enzymes, UGT enzymes and drug transporters in human kidney cortex samples. Data also include one adhesion protein and one plasma membrane marker. Protein expression is presented as a median, mean ± standard deviation (SD), % coefficient of variation (% CV) and range (min – max). Abundance of enzymes is expressed in pmol/mg of kidney microsomal protein.

Enzyme/Transporter	Median (pmol/mg protein)	Mean ± SD (pmol/mg protein)	CV (%)	Range (min – max) (pmol/mg protein)	n[a]
Cytochrome P450 enzymes					
CYP2B6	0.2	0.2 ± 0.02	10	0.16 – 0.22	15
CYP3A5	0.1	0.1 ± 0.05	35	0.06 – 0.24	18
CYP4F2	0.2	0.2 ± 0.1	51	0.13 – 0.48	16
Uridine-5'-diphospho-glucuronosyltransferase enzymes					
UGT1A1	0.14	0.1 ± 0.01	9.9	0.10 – 0.16	14
UGT1A3	0.13	0.1 ± 0.1	42	0.06 – 0.24	8
UGT1A4	0.20	0.2 ± 0.1	37	0.10 – 0.35	7
UGT1A6	0.13	0.2 ± 0.3	108	0.05 – 1.11	20
UGT1A9	2.1	2.6 ± 2.5	95	0.34 – 8.31	13
UGT2B7	0.88	1.5 ± 2.1	136	0.13 – 8.67	18
Transporters					
P-gp	8.4	7.7 ± 4.2	55	0.26 – 17.7	18
MRP4	1.4	2.3 ± 2.3	100	0.34 – 6.6	8
OCT3	0.9	1.1 ± 0.7	66	0.04 – 2.37	7
OAT2	1.8	1.8 ± 0.4	23	1.3 – 2.7	7
MATE-1	6.2	7.7 ± 4.7	61	1.4 – 17	16
OATP4C1	4.5	5.7 ± 5.1	90	0.18 – 16.0	15
OST-α	1.0	1.2 ± 0.5	39	0.69 – 1.91	7
OST-β	1.7	1.7 ± 0.7	43	0.70 – 3.3	8
Adhesion Protein					
CDH23	1.3	1.46 ± 0.71	48	0.68 – 2.50	5
Plasma Membrane Protein					
ATP1A1	135	160 ± 100	63	32.4 – 344	13

[a] **number of kidney cortex samples where protein could be quantified**

Table 2: Correlations matrix of the abundance data of 14 transporters obtained by either targeted or global proteomics. The extent of correlation based on Spearman correlations (Rs) is indicated both as strong and significant (Rs > 0.80, P < 0.0001) with very limited scatter (R^2 > 0.50) or moderate correlation (Rs > 0.60, P < 0.02) with limited scatter (R^2 > 0.30). Non-significant correlations were not included (-). Rs, Spearman correlation coefficient; *P*, probability value; R^2, linear regression.

	P-gp	MRP2	MRP4	MRP6	OCT2	OAT1	OAT3	OAT4	SGLT1	SGLT2	MATE-1	OATP4C1	OST-α	OST-β
P-gp	1	–	Rs=0.93 P<0.0001 R^2=0.71	–	Rs=0.60 P=0.01 R^2=0.30	Rs=0.84 P<0.0001 R^2=0.50	–	–	Rs=0.66 P=0.007 R^2=0.46	–	–	Rs=0.78 P=0.003 R^2=0.66	–	–
MRP2		1	–	–	–	–	Rs=0.94 P=0.005 R^2=0.97	–	–	–	–	–	–	–
MRP4			1	–	–	–	–	–	Rs=0.91 P<0.0001 R^2=0.56	–	–	Rs=0.96 P=0.003 R^2=0.86	–	–
MRP6				1	–	–	Rs=0.69 P=0.02 R^2=0.94	–	–	–	–	–	–	–
OCT2					1	Rs=0.83 P<0.0001 R^2=0.96	–	–	–	–	–	–	–	–
OAT1						1	–	–	–	–	–	Rs=0.95 P<0.0001 R^2=0.80	–	–
OAT3							1	Rs=0.83 P=0.008 R^2=0.92	–	–	–	–	Rs=0.96 P=0.003 R^2=0.85	–
OAT4								1	–	Rs=0.93 P=0.001 R^2=0.95	–	–	–	–
SGLT1									1	–	–	Rs=0.87 P<0.0001 R^2=0.60	–	–
SGLT2										1	–	–	–	–
MATE-1											1	Rs=0.75 P=0.01 R^2=0.73	–	–
OATP4C1												1	–	–
OST-α													1	Rs=0.78 P=0.003 R^2=0.51
OST-β														1