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# Quantitative Proteomics in Translational Absorption, Distribution, Metabolism, and Excretion and Precision Medicine[S]

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***Abstract*——A reliable translation of in vitro and preclinical data on drug absorption, distribution, metabolism, and excretion (ADME) to humans is important for safe and effective drug development. Precision medicine that is expected to provide the right clinical dose for the right patient at the right time requires a comprehensive understanding of population factors affecting drug disposition and response. Characterization of drug-metabolizing enzymes and transporters for the protein abundance and their interindividual as well as differential tissue and**

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**cross-species variabilities is important for translational ADME and precision medicine. This review first provides a brief overview of quantitative proteomics principles including liquid chromatography–tandem mass spectrometry tools, data acquisition approaches, proteomics sample preparation techniques, and quality controls for ensuring rigor and reproducibility in protein quantification data. Then, potential applications of quantitative proteomics in the translation of in vitro and preclinical data as well as prediction of interindividual variability are discussed in detail with tabulated examples. The applications of quantitative proteomics data in physiologically based pharmacokinetic modeling for ADME prediction are discussed with representative case examples. Finally, various considerations for reliable quantitative proteomics analysis for translational ADME and precision medicine and the future directions are discussed.**

***Significance Statement*——Quantitative proteomics analysis of drug-metabolizing enzymes and transporters in humans and preclinical species provides key physiological information that assists in the translation of in vitro and preclinical data to humans. This review provides the principles and applications of quantitative proteomics in characterizing in vitro, ex vivo, and preclinical models for translational research and interindividual variability prediction. Integration of these data into physiologically based pharmacokinetic modeling is proving to be critical for safe, effective, timely, and cost-effective drug development.**

## I. Introduction

Drug development is a resource-intensive process that relies on a wide range of in vitro, preclinical, and clinical experiments, costing approximately 1.5 billion dollars over a period of 10–12 years. A poor translation of in vitro and preclinical data to humans and high interindividual variability in drug disposition and response pose significant challenges in drug development. Translational absorption, distribution, metabolism, and excretion (ADME) encompasses quantitative understanding and integration of in vitro, preclinical, and exploratory human drug disposition data to predict the right dose for the right patient at the right time toward precision medicine. Because drug disposition and response can be affected by genetic and nongenetic factors, precision medicine is considered a promising approach for safe and effective drug development and pharmacotherapy.

Although physiologically based pharmacokinetic (PBPK) modeling is emerging as a promising approach for predicting drug disposition using in vitro data, it requires a myriad of physiological data including protein abundance of drug-metabolizing enzymes and transporter (DMET) proteins in the in vitro models and tissues as well as their interindividual variability and differential tissue abundance. In vitro data are often generated using recombinant systems (e.g., Supersomes), cell lines or vesicles overexpressing transporters, and human-derived models (e.g., microsomes or cultured hepatocytes). However, these models differ in the protein abundance of DMET proteins from the corresponding human tissues (Xu et al., 2018). Similarly, a quantitative understanding of interspecies differences in DMET abundance is required for a reliable allometry scaling of drug disposition and tissue distribution data from preclinical models to humans, especially for interpreting drug toxicity data. For example, SGX-523, an investigational mesenchymal-epithelial transition factor inhibitor, was discontinued after the phase 1 clinical trials due to nephrotoxicity, which was not captured during the animal toxicity studies. SGX-523 is primarily metabolized by aldehyde oxidase (AO) in the human liver; however, human-specific AO isoform is poorly expressed in preclinical species, particularly in rats and dogs (Diamond et al., 2010; Basit et al., 2021). Similarly, the renal abundance of organic anion transporter 2 (OAT2) is >5-fold higher in mice as compared with humans and monkeys (Basit et al., 2019). Such species differences in DMET abundance often lead to discordance between preclinical and clinical data.

Quantitative proteomics has emerged as a crucial technique in the translational ADME and precision medicine. Typically, quantitative proteomics involves selective quantification of the proteotypic peptide(s) unique to the protein(s) of interest by liquid chromatography–mass spectrometry (LC-MS/MS), without the need for an antibody, thus making it a superior technique over conventional methods such as western

**ABBREVIATIONS**: ADME, absorption, distribution, metabolism, and excretion; AO, aldehyde oxidase; AQUA, absolute quantification; AUC, area under the plasma concentration-time curve; BCRP, breast cancer resistance protein; BSEP, bile salt export pump; CES, carboxylesterases; CKD, chronic kidney disease; $CL_{int}$, intrinsic clearance; CNV, copy number variation; DDA, data-dependent acquisition; DDI, drug-drug interaction; DIA, data-independent acquisition; DMET, drug-metabolizing enzymes and transporter; ER, estrogen receptor; EV, extracellular vesicle; $f_m$, fractional contribution of an enzyme in drug metabolism; $f_t$, fractional contribution of a transporter in drug transport; IT, ion trap; iTRAQ, isobaric tags for relative and absolute quantification; IVIVE, in vitro to in vivo extrapolation; $K_m$, substrate affinity; LC-MS/MS, liquid chromatography–mass spectrometry; m/z, mass-to-charge; MPPGL, milligram of protein per gram of liver tissue; MPS, microphysiological systems; MRM, multiple reaction monitoring; OAT2, organic anion transporter 2; OCT1, organic cation transporter 1; PBPK, physiologically based pharmacokinetic; PCA, principal component analysis; P-gp, P-glycoprotein; PK, pharmacokinetics; PQC, positive quality control; PRM, parallel reaction monitoring; PSAQ, protein standards for absolute quantification; PTM, posttranslational modification; Q, quadruple; QconCAT, quantitative concatemers; RAF, relative activity factor; REF, relative expression factor; SIL, stable isotope-labeled; SNP, single nucleotide polymorphism; SWATH, sequential windowed acquisition of all theoretical fragment ions; TMT, tandem mass tags; TOF, time-of-flight; TPA, total protein approach; UGT2B7, UDP-glucuronosyltransferase 2B7; $V_{max}$, velocity maximum.

blotting. The technique has been applied for characterizing DMET abundance for the assessment of (1) interindividual variability, i.e., the effect of age, genotype and sex, (2) differential tissue variability, (3) interspecies differences, and (4) the viability of in vitro models as illustrated with examples in Tables 1–5. Together, these data are important for in vitro to in vivo extrapolation (IVIVE) and PBPK modeling. For example, morphine and zidovudine pharmacokinetics (PK) and clearance were predicted in the neonatal population using ontogeny data of UDP-glucuronosyltransferase 2B7 (UGT2B7) and organic cation transporter 1 (OCT1) in pediatric and adult human liver samples (Emoto et al., 2017; Emoto et al., 2018; Bhatt et al., 2019). Similarly, the effect of disease states and genetic variations on drug PK and clearance have been successfully predicted by integrating quantitative proteomics data in PBPK modeling (Emoto et al., 2017; Bhatt et al., 2019; Vildhede et al., 2020). Moreover, quantitative proteomics data have also been shown to predict the fractional contribution of individual enzymes ($f_m$) and transporters ($f_t$) in drug disposition and drug-drug interaction (DDI) (Jamei et al., 2014; Li et al., 2019a; Anoshchenko et al., 2020; Kimoto et al., 2020). Similarly, the technique has been used for the selective quantification of biomarkers for monitoring drug response and disease diagnosis (Heo et al., 2007; Warth et al., 2012; Basit et al., 2020) and for identifying nonsynonymous single nucleotide polymorphism (SNPs) and post-translational modification (PTMs) (Shi et al., 2018b). Further, the selectivity of quantitative proteomics results in high signal-to-noise ratio for a sensitive determination of DMET abundance in a small sample volume (e.g., 24-hepatocytes on-column, and 10 ng microsomal protein on-column) (Ahire et al., 2021).

Here, we provide a summary of various MS techniques, acquisition methods, and sample preparation approaches used in quantitative proteomics, with a detailed compilation of the reported applications of the technique in the in vitro model characterization, IVIVE, interindividual variability, intertissue differences, interspecies variability, PBPK modeling, and drug response prediction. Various considerations and recommendations for a robust quantitative DMET proteomics analysis, including the future directions, are also discussed.

## II. Quantitative Proteomics: Basic Principles

Quantitative proteomics has emerged as a major approach for protein quantification due to the significant developments in both LC-MS hardware and software. The technique is primarily based on the bottom-up or shotgun proteomics that relies on the analysis of digested proteins by a protease enzyme(s) (e.g., trypsin, trypsin plus Lys-C, chymotrypsin, or proteinase C) in biologic samples. Due to the limitations of the spectral counting method of protein quantification (Lundgren et al., 2010), chemical and metabolic labeling approaches were developed in the late 1990s for relative and absolute protein quantification (Fig. 1A). The labeling techniques include isotope-coded affinity tag (Gygi et al., 1999), stable isotope labeling by amino acids in cell culture (SILAC) (Ong et al., 2002), tandem mass tags (TMT) (Thompson et al., 2003), and isobaric tags for relative and absolute quantification (iTRAQ) (Wiese et al., 2007). The labeling methods provided advantages of greater accuracy and precision but suffer from limitations of multistep sample preparation, higher cost, and higher sample requirement. Recent approaches like absolute quantification (AQUA) or multiple reaction monitoring (MRM) (Gerber et al., 2003), quantitative concatemers (QconCAT) (Beynon et al., 2005; Carroll et al., 2011), protein standards for absolute quantification (PSAQ) (Kaiser et al., 2011), and stable isotope labeling and capture by anti-peptide antibodies (SISCAPA) address some of these limitations (Beynon et al., 2005).

### *A. Liquid Chromatography–Mass Spectrometry Techniques and Data Acquisition Approaches*

The LC separates analytes based on lipophilicity, and the MS measures the mass-to-charge (m/z) ratio of ions in the gas phase. In the MS, an ion source first ionizes analytes in the gas phase, ions are separated in a mass analyzer(s) based on the m/z ratio, and the product ion intensity is recorded by a detector. The breakthrough of electrospray ionization in the 1990s solved the problem of ion generation from large and nonvolatile analytes (e.g., proteins and peptides), making it suitable for liquid chromatography. Atmospheric pressure chemical ionization has also been used in the proteomics study, especially for analysis of smaller peptides, albeit to a lesser extent than electrospray ionization. Atmospheric pressure chemical ionization is a softer ionization technique in which the ionization of analytes is performed by proton transfer at atmospheric pressure outside of the vacuum chamber in the MS (Doerge et al., 1996; Toribio et al., 2000).

Typical mass analyzers used in proteomics are, (1) quadrupole (Q), (2) ion trap (IT), (3) time-of-flight (TOF), (4) orbitrap, and (5) Fourier-transform ion cyclotron resonance. Although the analyzers can be used standalone, hybrid instruments are designed to combine the unique capabilities of mass analyzers for greater analytical performance (Supplemental Table 1). The hybrid MS analyzers include triple quadrupole, hybrid quadrupole ITs (Q-q–linear IT or Q-trap), Q-TOF, TOF-TOF, LTQ orbitrap, and LTQ Fourier-transform ion cyclotron resonance.

MS-based data acquisition falls under two major categories: targeted (hypothesis-driven) and untargeted (hypothesis-generating) approaches (Fig. 1B). The selection of the MS acquisition approach depends

TABLE 1
Successful examples of applications of quantitative proteomics for characterization of in vitro ADME models

| Organ | Study Objective | Target DMET Protein Quantified | Reference |
|---|---|---|---|
| Liver | Effect of pregnancy related hormones on protein abundance of UGTs in primary human hepatocytes | UGTA1 and UGT2B7 | Khatri et al., 2021 |
| | Hepatocyte model selection to predict the uptake and biliary efflux of drugs in suspended, plated, and sandwich-cultured human hepatocytes | OATP1B1, NTCP, MRP3, and BSEP | Kumar et al., 2019 |
| | Characterization of HepG2 3D spheroid model for hepatotoxicity | CYPs, UGTs, GSTs, MRP1, and MRP4 | Hurrell et al., 2019 |
| | Comparison of protein abundance between human liver microsomes and hepatic cell lines (e.g., HepG2, Hep3B, and Huh7) | CYPs, UGTs, SULTs, AKRs, GSTs, ALDHs, ADHs, AO, CESs, PONs, and EPXs | Shi et al., 2018a |
| | Comparative proteomics analysis of HLM and HLS9 fractions for in vitro drug metabolism studies | CYPs, UGTs, SULTs, AKRs, GSTs, ALDHs, ADHs, AO, CESs, PONs, and EPXs | Wang et al., 2020 |
| | Effect of pregnancy-related hormones and induction of CYP3A activity over the course of pregnancy in HepaRG and SCHHs cells | CYP3A | Zhang et al., 2015b |
| | Test primary hepatocytes as a suitable model for hepatic drug metabolism study | CYPs, UGTs, MDR1, MRPs, BCRP, NCP, OCTs, OATPs, CNTa, ENT1, and ATE1 | Schaefer et al., 2012 |
| | Interindividual variability in DMEs in HLMs | CYPs and UGTs | Ohtsuki et al., 2012 |
| | Compare protein abundance between hepatic cell lines (HepG2, Hep3B, and Huh7) | CYPs, UGTs, and ALDHs | Shi et al., 2018a |
| Intestine | Drug metabolizing capabilities of human primary intestinal monolayers | CYP3A4, UGT1A1, UGT1A10, UGT2B17, and CES2 | Speer et al., 2019 |
| | Effect of culture duration on transporter abundance in Caco-2 cells | MDR1, BCRP, PEPT1, OST$\alpha$ and OST$\beta$, MRP2, MRP4, OATP2B1 and MCT1 | Uchida et al., 2015 |
| | Regional DMEs protein changes along the line of the small intestine using cryopreserved human intestinal mucosa (CHIM) model | UGTs, CESs, and SULTs | Zhang et al., 2020a |
| Brain | Determine the protein abundance of transporters and receptors in human brain microvessels | BCRP and P-gp | Uchida et al., 2011 |
| | Protein abundance of key membrane proteins in hCMEC/D3 cells and human brain microvessels | ABCA2, MDR1, MRP4, BCRP, GLUT1, 4F2hc, MCT1, and ENT1 | Ohtsuki et al., 2013 |
| | Identify DMET protein expression in brain microvascular endothelial cells | GLUT1, EAAT1, EAAT2, BCRP, MDR1, CYP1B1, CYP2U1, GSTP1, COMT, GSTM3, GSTO1, and GSTM2 | Shawahna et al., 2011 |
| | Quantification of brain transporter abundance in brain microvascular endothelial cells | BCRP, P-gp, OATP2B1, ENT1, and GLUT1 | Billington et al., 2019 |
| Skin | Characterization of in vitro models of human skin | CYPs, ADHs, ALDHs, AKRs, AO, CESs, EPHx, and PONs | Couto et al., 2021 |
| Eye (retina) | Transporter protein abundance in the plasma membranes of the human retinal pigment epithelium cells and immortalized arpe19 cell line) | MRP1, MRP5, GLUT1, 4F2hc, TAUT, CAT1, LAT1, MPR7, OAT2, MCT1, MCT4, MRP4, RFC1, and MATE1 | Pelkonen et al., 2017 |

on the experimental goals, such as the need for proteome coverage versus sensitivity and precision (Elias et al., 2005; Pino et al., 2020). For example, a selected list of DMET proteins can be quantified by the targeted approaches such as MRM or parallel reaction monitoring (PRM) for sensitive and precise quantification. On the other hand, the untargeted acquisition approaches [e.g., data-dependent acquisition (DDA) and data-independent acquisition (DIA)] are employed for achieving a dynamic peptide coverage. DDA method acquires MS ions based on their intensities in each sample (e.g., top 10), whereas DIA acquires all theoretical ions irrespective of their intensities for deeper proteome coverage that is possible with higher data acquisition speed. In general, targeted proteomics methods are more sensitive and precise for a small number of proteins, whereas untargeted proteomics is superior for obtaining proteome-wide coverage and simultaneous quantification of a large number of proteins, thus providing information at biologic pathway levels. A detailed comparison of different protein acquisition approaches and conventional immunoquantification is provided in Supplemental Table 2 and summarized below.

*1. Targeted Proteomics.* MRM is the most popular technique for DMET proteomics analysis, in which proteotypic or surrogate peptide(s) unique to the target protein is quantified (Fig. 1C) (Wolf-Yadlin et al., 2007; Kamiie et al., 2008). MRM is performed on a triple quadrupole or Q-trap mass spectrometers, where the first quadrupole is set to allow only a selected m/z ratio of a precursor ion into the second quadrupole. The MS fragmentation of the selected ion by collision-induced dissociation in the second quadruple yields characteristic product ions. The product ions are filtered in the third quadrupole and detected by a MS detector (e.g., photomultiplier tube). Generally, the top 3 high intensity product ions are monitored for a reliable peptide quantification (Lange et al., 2008; Vidova and Spacil, 2017). Although MRM-based proteomics is selective and precise, it can only quantify a limited number of product ions (10–100) concurrently. Scheduled or dynamic MRM partially addresses this problem by analyzing target ions in a prefixed elution time window, thus allowing analysis

TABLE 2
Successful examples of the application of quantitative proteomics for the characterization of interindividual variability in DMET abundance

| Enzyme/Transporter | Fold Change | Reference |
|---|---|---|
| | Effect of age (liver): fold change (relative to infants) | |
| CYP1A2 | >2-fold (adults); >1- to 2-fold (juveniles) | Sadler et al., 2016 |
| CYP2A6 | >2-fold (adults) | |
| CYP2B6 | >2-fold (juveniles) | |
| CYP3A5 | >2-fold (juveniles, adults) | |
| UGT1A1 | >1- to 2-fold (adults) | |
| UGT1A4 | >2-fold (children, adolescents, adults) | Bhatt et al., 2019 |
| UGT1A6 | >2-fold (children, adolescents, adults) | |
| UGT1A9 | >2-fold (adults) | |
| UGT2B7 | >2-fold (adolescents, adults) | |
| UGT2B15 | >2-fold (adults) | |
| UGT2B17 | >2-fold (children, adolescents, adults); | Bhatt et al., 2018 |
| ALDH1A1 | >1- to 2-fold (children, adolescents) | Bhatt et al., 2017 |
| ADH1B | >2-fold (children); >1- to 2-fold (adolescents, adults) | |
| ADH1C | >2-fold (children, adolescents, adults) | |
| CES1 | >2-fold (adults); >1- to 2-fold (children, adolescents) | Boberg et al., 2017 |
| FMO3 | >1- to 2-fold (children, adult) | Zane et al., 2018 |
| SULT1A1 | >1- to 2-fold (children) | Ladumor et al., 2019a |
| SULT1A2 | >1– to 2-fold (adolescents, adults) | Dubaisi et al., 2019 |
| SULT1B1 | >2-fold (infants, children, adolescents, adults) | Ladumor et al., 2019a) |
| OCT1 | >1- to 2-fold (adolescents, adults) | Prasad et al., 2016; van Groen et al., 2018 |
| OATP1B3 | >1- to 2-fold (children, adolescents, adults) | Prasad et al., 2016; van Groen et al., 2018 |
| P-gp | >1- to 2-fold (adolescents, adults) | Prasad et al., 2016; van Groen et al., 2018 |
| MRP3 | >1- to 2-fold (adult) | Prasad et al., 2016; van Groen et al., 2018 |
| | Effect of age (kidney): fold change (relative to infants) | |
| P-gp | >1- to 2-fold (children, adults) | Cheung et al., 2019 |
| URAT1 | >2-fold (children) | |
| OAT1 | >1- to 2-fold (children, adults) | |
| OAT3 | >1- to 2-fold (children, adolescents, adults) | |
| OCT2 | >1- to 2-fold (children) | |
| | Effect of genotype: fold change (relative to the corresponding reference allele) | |
| BCRP | <0.5-fold (rs2231142 (C421A)) | Prasad et al., 2013 |
| MRP2 | >1- to 2-fold (SNP 21214G>A (V417I) | Deo et al., 2012 |
| OATP1B1 | >2-fold (SNP c.463C>A); >1- to 2-fold (388 A>G, c.597C>T) | Prasad et al., 2014 |
| CYP2A6 | >1- to 2-fold (*1/*4, ↑ *1/*9); <1–0.5-fold (*1/*17, *1/*2) | Tanner et al., 2017; Tanner et al., 2018 |
| FMO3 | >1- to 2-fold (E158K:G308G) | Xu et al., 2017 |
| UGT2B17 | >1- to 2-fold (*1/*2); < 0.5-fold (*2/*2) | Gaedigk et al., 2018 |
| | Effect of sex: fold change (male versus female adults) | |
| BCRP | >1- to 2-fold | Prasad et al., 2013 |
| UGT2B17 | >2-fold | Tanner et al., 2017 |
| CYP2A6 | <1- to 2-fold | |
| FMO3 | >1- to 2-fold | Xu et al., 2017 |
| | Effect of disease condition: fold change (relative to healthy adults) | |
| Alcoholic liver disease (CPS A) | >2-fold (P-gp); < 0.5-fold (BSEP, MRP2, OAT2, OATP1B1, OATP2B1) | Wang et al., 2016; Drozdzik et al., 2020 |
| Alcoholic liver disease (CPS B) | >2-fold (P-gp); <0.5-fold (BSEP, MRP2, NTCP, OCT1, OCT3, OAT2, OATP1B1, OATP1B3, OATP2B1) | Drozdzik et al., 2020 |
| Alcoholic liver disease (CPS C) | >2-fold (P-gp, MRP3, BCRP, OCT3); <0.5-fold (MRP2, NTCP, OAT2, OATP1B1, OATP2B1) | Drozdzik et al., 2020 |
| Nonalcoholic fatty liver disease | <0.5-fold (CYP3A4) | Ali et al., 2017 |
| Nonalcoholic steatohepatitis | >2-fold (OATP1B1, MRP4); <0.5-fold (NTCP, OATP1B3) | |
| HCV | >2-fold (MRP3, MRP4, P-gp); <0.5-fold (CYP3A4, CYP2C9, CYP2E1, CYP1A2, CYP2A6, CYP2C8, UGT1A4, UGT1A6, UGT2B7, UGT2B15, CES1, ADH1B, ADH1C) | Wang et al., 2016 |
| HCV (CPS A) | >2-fold (P-gp); <0.5-fold (BSEP, MRP1, MRP2, BCRP, NTCP, OCT1, OCT3, OATP1B1, OATP2B1) | Drozdzik et al., 2020 |
| HCV (CPS B) | >2-fold (P-gp, MRP1); <0.5-fold (BSEP, MRP2, OCT1, OCT3, OATP1B3, OATP2B1) | |
| HCV (CPS C) | >2-fold (P-gp, MRP3); <0.5-fold (BSEP, MRP1, NTCP, OCT1, OCT3, OATP1B3, OATP2B1) | |
| Primary biliary cirrhosis; PSC (CPS A) | | |

(*continued*)

TABLE 2—*Continued*

| Enzyme/Transporter | Fold Change | Reference |
|---|---|---|
| | >2-fold (P-gp, BSEP, MRP3, OCT3, OATP1B3); <0.5-fold (MRP2) | |
| PSC (CPS B) | >2-fold (P-gp, MRP3, OCT3); <0.5-fold (MRP2, OATP1B3) | |
| Autoimmune hepatitis (AIH) (CPS A) | >2-fold (P-gp, MRP3, BCRP, OCT3); <0.5-fold (MRP1, MRP2) | |
| AIH (CPS B) | >2-fold (P-gp, MRP3, BCRP, OCT3); <0.5-fold (MRP2) | |
| AIH (CPS C) | >2-fold (P-gp, MRP3, OCT3); <0.5-fold (MRP2, NTCP, OCT1, OATP1B1) | |
| Ulcerative colitis | <0.5-fold (MCT1) | Erdmann et al., 2019 |

of >300 transitions per 30 minutes (Carr et al., 2014; Ronsein et al., 2015). PRM or MRM high resolution provides a more advanced alternative due to its ability to quantify all fragment ions from a single or multiple precursor ions simultaneously (Fig. 2) (Peterson et al., 2012). PRM offers advantages such as the ability to quantify 100s–1000s of proteins, higher specificity, and high-throughput quantification (Peterson et al., 2012; Kim et al., 2016) without the need for additional method development and optimization efforts (Rauniyar, 2015). Skyline (Genome Sciences, University of Washington, Seattle, WA) is currently the most used open-access software for targeted proteomics method development and analysis.

Proteotypic or surrogate peptide selection is the first step in both MRM and PRM (Kamiie et al., 2008). Briefly, the proteotypic peptides should be unique, have an optimum length of 7–22 amino acids, should be devoid of transmembrane region, and should not be prone to PTM or change by a SNP site. Ideally, unstable amino acid residues (e.g., C, M, W), ragged ends (RR, KK, RK, and KR), or potential missed cleavage sites are avoided. The peptide should also have an optimum hydrophobicity (~30% hydrophobic residues) to be retained on a chromatographic column (Fig. 2). In MRM, a synthetic stable isotope-labeled (SIL) or heavy peptide with $^{13}C$ and/or $^{15}N$-labeling at the carboxyl-terminal of lysine or arginine residues are used as internal standards to normalize the matrix effect (MS ion suppression) and other postdigestion variables (Lange et al., 2008). Alternatively, an external multipoint calibration curve of a standard light peptide can be used for absolute peptide quantification, especially when the linearity is warranted over a large dynamic range.

*2. Untargeted Proteomics.* Untargeted proteomics is a hypothesis-independent approach that utilizes high-resolution MS data in conjunction with bioinformatics tools such as MaxQuant (Max Planck Institute of Biochemistry, Germany) for unbiased system-wide proteome identification and quantification (100s–1000s of proteins). Untargeted proteomics data are acquired using one of the two techniques, i.e., DDA and DIA. In DDA, full-scan mass spectrum determines the number of peptides present in a sample, and the most abundant precursor ions (e.g., Top 10) are selected and fragmented in the second stage. Although DDA is selective for high-intensity peptides, it is not efficient in the quantification of low-abundant proteins (Meyer, 2019; Smith et al., 2019; Tiwary et al., 2019). Further, each peptide species in DDA is purposefully detected only a few times, resulting in limited precision. On the other hand, DIA is a more advanced approach that allows for the detection of all theoretical masses in the preselected m/z windows, which are fragmented and analyzed to record all theoretical second stage scans (Reubsaet et al., 2019). DIA generally involves one of the two approaches: sequential windowed acquisition of all theoretical fragment ions (SWATH) and collision energy alteration (LC-$MS^E$). In SWATH, the full mass range is divided into several smaller m/z windows, and all precursor ions in a preselected m/z isolation window are fragmented (Ludwig et al., 2018; Smith et al., 2019). During each cycle time (2–4 seconds), the precursor ions are selected and fragmented multiple times, providing time-resolved product ions of all eluted precursor ions. In $MS^E$, the precursor and product ions are analyzed at low- and high-collision energy scans, and the precursor ions are fragmented within a selected m/z range. DDA and DIA data are generally analyzed using open-access software, e.g., MaxQuant and OpenSWATH.

### *B. Protein Quantification Approaches*

Protein quantification can be achieved utilizing either labeled-based or label-free approaches. The first approach is based on chemical or metabolic labeling for a sensitive and reproducible relative or absolute quantification. For example, in stable isotope labeling by amino acids in cell culture, the heavy amino acids (lysine, arginine, or both) are introduced into the proteins in the cell culture for one condition (e.g., test), whereas the cells are grown in regular media for another condition (control). The harvested cells are mixed and homogenized before digestion (Ong et al., 2002). The light to heavy peptide signal ratio provides an estimate of quantitative changes in test versus control. Isotope-coded affinity tag utilizes chemical labeling of cysteine residues (Gygi et al., 1999), whereas in iTRAQ and TMT, the digested peptides are labeled

TABLE 3
Common single nucleotide polymorphism in DMET proteins that affect their activity or abundance

| Protein | Nucleotide Change; Allele; Amino Acid Change (rs Number) | MAF (Caucasians, %) | Activity (Abundance) | Clinical Application | References |
|---|---|---|---|---|---|
| CYP1A1 | 3801 T>C; *2A; I462V (rs4646903) | 19 | Increased (unknown) | Predictor for clinical outcome in liver cancer patients treated with EGFR-TKI therapy | Zhou et al., 2009 |
| CYP1A2 | −163C>A; *1F; NA (rs762551) | 33.3 | Increased (increased) | Associated with the risk for colorectal adenomas in humans | Sachse et al., 2003; Moonen et al., 2005; Pilgrim et al., 2012 |
| | −246T>delT; *1D; NA | 4.82 | Decreased (decreased) | Higher olanzapine plasma concentration | Sachse et al., 2003; Uslu et al., 2010; Czerwensky et al., 2015 |
| CYP2C9 | 430 C>T; *2; R144C (rs1799853) | 19.0% *1/*2 1.6% *2/*2 1.8% *2/*3 | Decreased (unknown) | Poor warfarin metabolism | Aithal et al., 1999; Hillman et al., 2004; Lindh et al., 2009 |
| | 1075A>C; *3; I359L (rs1057910) | 9 | Decreased (unknown) | Decrease metabolism of phenytoin compared with reference genotype | Scordo et al., 2001; van der Weide et al., 2001; Rosemary et al., 2006 |
| CYP2C19 | 681G>A; *2; splicing variant I331V (rs4244285) | 16 | Decreased (unknown) | In Asians with ulcer treatment of Helicobacter pylori infection with omeprazole varying treatment effectiveness | Aynacioglu et al., 1999 |
| | −806C>T and -3402C>T; *17; I331V (rs12248560) | 18 | Increased (increased) | Increase clinical response to clopidogrel treatment by better antiplatelet activity and higher risk of bleeding | Sibbing et al., 2010; Santos et al., 2011 |
| CYP2D6 | 1749A>G, 2549delA; *3; frameshift mutation, N166D; 259 (rs1135824) | 2.04 | Decreased (decreased) | Decreased metabolism of risperidone | Sachse et al., 1997 |
| | 1846G>A; *4; P34S; L91M; H94R; Splicing variant; S486T (rs3892097) | 20.7 | Decreased (decreased) | Poor metabolism of tricyclic antidepressants may lead to increased toxicity in Caucasians | Sachse et al., 1997; Bijl et al., 2008 |
| | 100C>T; 1039C>T; 1661G>C; 1846G>A; 4180G>C; *4D; P34S; Splicing variant; S486T (NA) | 3.4 | Decreased (decreased) | | Shimada et al., 2001 |
| | 100C>T; 997C>G; 1661G>C; 1846G>A; 4180G>C; *4L; P34S; Splicing variant; S486T (NA) | 4.5 | Decreased (decreased) | | Shimada et al., 2001 |
| | whole gene deletion; *5; CYP2D6 deleted (NA) | 4.1 | Deletion (deleted) | Require dose adjustment of CYP2D6 substrates | Griese et al., 1998 |
| | 1707delT; *6; frameshift mutation, 118F (rs5030655) | 1.3 | Nonfunctional (decreased) | Person may experience haloperidol-induced extrapyramidal side effects | Griese et al., 1998; Šimić et al., 2016) |
| | 2935A>C; *7; H324 (rs5030867) | 1 | Decreased (unknown) | | Evert et al., 1997 |
| | 2615-2617delAAG; *9; K281del (rs5030656) | 2 | Decreased (unknown) | | Griese et al., 1998 |
| | 100C>T; *10; P34S; S486T (rs1065852) | 8 | Decreased (unknown) | | Griese et al., 1998; Raimundo et al., 2004 |
| | 2988G>A; *41; R296C; Splicing variant; S486T (rs28371725) | 8 | Decreased (decreased) | | Raimundo et al., 2004 |
| CYP2A6 | NA; *12; substitutions 10aa (esv2663194) | 2.9 | Decreased (unknown) | | Haberl et al., 2005 |
| | NA; *1B; NA;NA | 32.6 | Increased (unknown) | Faster nicotine metabolism | Mwenifumbo et al., 2008; Djordjevic et al., 2013 |
| | 1799 T>A; *2; L160 (rs1801272) | 2.3 | Decreased (unknown) | Shifting of coumarin metabolism from 7-OH coumarin to 2-hydroxyphenylacetic acid | Hadidi et al., 1997; Ingelman-Sundberg, 2001 |

(*continued*)

TABLE 3—*Continued*

| Protein | Nucleotide Change; Allele; Amino Acid Change (rs Number) | MAF (Caucasians, %) | Activity (Abundance) | Clinical Application | References |
|---|---|---|---|---|---|
| | NA; *4; deletion | | Deletion (deleted) | Impairment of nicotine metabolism | Ingelman-Sundberg, 2001 |
| | − 48 T > G; *9; TATA box (rs28399433) | 7.1 | Decreased (decreased) | | Schoedel et al., 2004 |
| CYP3A4 | 566T>C; *17; F189S (rs4987161) | | Decreased (unknown) | Decreased metabolism of nifedipine | Dai et al., 2001 |
| | NA; *1B; NA (rs2740574) | 17 | Increased (unknown) | Higher docetaxel metabolism | Kadlubar et al., 2003; Tran et al., 2006 |
| | 664T>C; *2; S222P (rs5585340) | 2.7 | Decreased (unknown) | | Sata et al., 2000 |
| CYP3A5 | 6986A>G; *3C; Splicing defect (rs776746) | 81.3 | Decreased (decreased) | Prognosis of non–small cell lung cancer patients undergoing chemotherapy and surgery | Quaranta et al., 2006; Jiang et al., 2016 |
| | 6981A>G, 29748T>C; *3k / *10; Splicing defect; F446S (rs41279854) | 2.0 | Decreased (decreased) | | Lee et al., 2003 |
| CYP2B6 | 64C>T; *2; R22C; rs8192709 | 5.3 | Increased (unknown) | | Hiratsuka et al., 2002; Honda et al., 2011 |
| | 1459C>T; *5; R487C (rs3211371) | 14.0 | Decreased (unknown) | Decreased S-mephenytoin N-demethylase activity | Lang et al., 2001 |
| | 785A>G; *4; K262R (rs4802101) | 5.0 | Increased (unknown) | | Lang et al., 2001; Kirchheiner et al., 2003 |
| | 516G>T, 785A>G; *6; Q172H; K262R (rs3745274, rs2279343) | 25.2 | Decreased (unknown) | | Lang et al., 2001; Kirchheiner et al., 2003 |
| | −1456T>C, -750T>C, .785A>G, 1459C>T; *7; Q172H; K262R; R487C (rs3745274, rs2279343, rs3211371) | | Decreased (unknown) | Decreased S-mephenytoin N-demethylase activity | Hiratsuka et al., 2002; Lamba et al., 2003 |
| | Gene deletion, *2; NA (rs10025771) | | Decreased (decreased) | | Bhatt et al., 2018 |
| UGT2B17 | −155G>A,840A>T, 788G>T, 2603T>G; H2/H2; NA (rs7436962, rs9996186, rs4860305, and rs28374627) | 22 | Increased | | Bhatt et al., 2018 |
| UGT1A1 | NA; *28; TATA box (rs8175347) | 26–31 | Decreased (decreased) | Decreased glucuronidation of bilirubin to about 30% of wild-type levels, adverse reactions to irinotecan treatment | Bosma et al., 1995; Marques and Ikediobi, 2010 |
| SULT2A1 | 187G>C, 679A>G, 781G>A; NA; A63P, K227D and A261T (rs6639786, rs2270112, and rs17268988) | | Decreased (NA) | Decreased metabolism of tibolone | Thomae et al., 2002; Miller et al., 2018; Wong et al., 2018 |
| GSTP1 | 313A>G; NA; I105V (rs1695) | 0.08–0.33 | Decreased (unknown) | Alters the pharmacokinetics of cyclophosphamide | Sharma et al., 2014 |
| UGT2B7 | 802C>T; *2; H268Y (rs7439366) | 50 | Decreased (NA) | Higher exposure of epirubicin | Bhasker et al., 2000; Parmar et al., 2011; Uchaipichat et al., 2013 |
| UGT2B15 | 253G>**T; *2;** D85Y (NA) | 50 | Decreased (NA) | Interindividual variability in the clearance of oxazepam and lorazepam | |
| BCRP | 421C>A; NA; Q41K (rs2231342) | 4.5–12 | Decreased (decreased) | AUC of sulfasalazine, simvastatin, and rosuvastatin >2-fold higher in comparison with reference allele | Prasad et al., 2013; Tanaka et al., 2015 |

(*continued*)

TABLE 3—*Continued*

| Protein | Nucleotide Change; Allele; Amino Acid Change (rs Number) | MAF (Caucasians, %) | Activity (Abundance) | Clinical Application | References |
|---|---|---|---|---|---|
| OATP2B1 | 388A>G, 521T>C and 388A>G; c.521T>C, Asn130Asp; p.Val174Ala; *1b, *5, and *15; N130D, V174A, (rs2306283, rs2306283) | 30, 16 and 14 | Decreased (NA) | Interindividual variability in statin therapy | Gao et al., 2017; Mori et al., 2019 |
| OATP1B1 | 463C>A; *14; R155C (rs149535236) | 2 | Increased (increased) | Cerivastatin-induced rhabdomyolysis | Couvert et al., 2008; Romaine et al., 2010; Tamraz et al., 2013 |
| OCT1 | 181C>T, 1201G>A, 1393G>A, 1257delATG; *2, *3, *4, *5; R61C, G401S, G465R and deletion of M420 (rs12208357, rs34130495, rs34059508, rs35167514) | 9, 3, 16 | Decreased (NA) | Impaired metformin uptake and lower morphine clearance in pediatrics | Saadatmand et al., 2012; Fukuda et al., 2013; Arimany-Nardi et al., 2015 |
| OCT2 | 808G>T; NA; A270S (rs316019) | | NA (NA) | | Meyer et al., 2017) |

by chemical tags at the free amines (Gygi et al., 1999; Thompson et al., 2003). The labeled samples are mixed for multiplexed analysis by targeted or untargeted proteomics approaches. Because the tags are isobaric in iTRAQ and TMT, no mass shift is detected in the labeled peptides in the full parent ion scan, and the signals from a single peptide from all samples are summed, whereas the distribution of isotopes in the different tags represents the tag-specific "reporter" ion intensity. The ratio of MS signal intensities from the tags provides the relative amounts of the peptide in different samples (Wiese et al., 2007).

Isotope dilution is another approach that utilizes spiked-in SIL peptide standards (Stöcklin et al., 1997; Brun et al., 2007). A known and consistent amount of SIL peptides is spiked into all samples, and the ratio is monitored to estimate the absolute levels. However, isotope dilution assay can only be used for a limited number of proteins because of the cost (Li et al., 2016; Vildhede et al., 2018; Wiśniewski et al., 2019). AQUA, QconCAT, and PSAQ all use the isotope dilution or spiked-in standards, where AQUA standards consist of synthetic peptides that are spiked into the sample after proteolysis (Gerber et al., 2003), QconCAT concatemers are chimeric proteins that are composed of different proteotypic peptides from multiple target proteins (Beynon et al., 2005), and PSAQ standards are full-length protein(s) that match the biochemical properties of the target protein(s) and spiked in at the beginning of the analytical process (Kaiser et al., 2011).

The total protein approach (TPA) is an emerging global quantitative proteomics strategy that utilizes a computational method to convert spectral intensities to protein concentrations (Vildhede et al., 2018). First used in 2012 (Wiśniewski et al., 2012), TPA has been applied in protein quantification for a variety of applications in ADME research (Wiśniewski and Rakus, 2014; Bryk and Wiśniewski, 2017; Ölander et al., 2020). TPA is centered on the quantification of individual proteins and groups of functionally related proteins in any protein mixture without the use of spike-in standards. Thus, TPA protein quantification approach can be applied to diverse large-scale proteomic data with reasonable precision. However, TPA is reliant on the depth of the proteomic analysis, which, in theory, requires complete digestion of all proteins in a sample using more than one protease to achieve precise protein quantification (Wiśniewski et al., 2019).

### *C. Proteomics Sample Preparation Approaches*

Efficient and reproducible sample preparation is the key to quantitative proteomic analysis. Although many sample preparation approaches are used for the reproducible quantification of proteins (Supplemental Table 3), DMET proteins pose unique challenges in the sample preparation because of the transmembrane nature of DMET proteins. Quantitative proteomics analysis of transmembrane proteins is often challenging because these proteins are coagulated during the protein precipitation step, making them non-accessible to the proteolytic enzymes. To achieve optimum digestion, membrane proteins require additional treatments such as membrane solubilization or enrichment using immunoprecipitation, centrifugation, or cell surface biotinylation. Membrane solubilization utilizes MS-compatible detergents, organic solvents, and organic acids (Han et al., 2001; Blonder et al., 2002) to make them more accessible for proteolysis. Plasma membrane enrichment by surface biotinylation and enrichment via streptavidin beads is another method that reduces the contamination from other subcellular organelles and enhances the quantification efficiency (Shin et al., 2003; Zhang et al., 2003; Zhao et al., 2004; Kumar et al., 2017b). For example, the biotinylation approach has been used to enrich the apical and basolateral membrane transporters of rat kidney collecting ducts before LC-MS/MS analysis

TABLE 4
Successful examples of proteomics informed PBPK modeling and IVIVE for predicting drug disposition

| Drugs | Enzymes and/or Transporters Involved | Study Objective | Study Population | Predictive Accuracy (P/O fold ratio) | Software Used | References |
|---|---|---|---|---|---|---|
| Zidovudine | UGT2B7 | Liver cirrhosis | Adult Child Pugh C subjects | 0.97 | Simcyp | Prasad et al., 2018 |
| | | Age-dependent PK | Neonates, infants and children | 0.5–2* | | Bhatt et al., 2019 |
| Morphine | UGT2B7 and OCT1 | Liver cirrhosis | Adult Child Pugh C subjects | 0.97 | Simcyp | Prasad et al., 2018 |
| | | Age-dependent PK | Neonates, infants and children | 0.5–2* | | Prasad et al., 2018 |
| Acetaminophen | SULT1A1/1A3/ 1B1/2A1 | Age-dependent PK and its effect on fraction of drug metabolized | Neonates and infants | 0.98 | GastroPlus | Ladumor et al., 2019a |
| Rosuvastatin | OATP1B1/1B3/ 2B1, NTCP | Effect of body weight on rosuvastatin PK | Adult healthy subjects with varying body weights | 0.95 | Semimechanistic model using R | Wegler et al., 2021 |
| Lamotrigine | UGT1A3/1A4/2B7 | Alcoholic cirrhosis | Adult hepatic impaired subjects | 0.83 | GastroPlus | Ladumor et al., 2019b |
| Oseltamivir | CES1/2 | Age-dependent PK | Infants | 0.5–2.1* | Simcyp | Boberg et al., 2017 |
| Ethionamide | FMO3 | Drug-drug interaction with methimazole and impact of genetic polymorphism | Healthy subjects | 0.6–2.6* | Simcyp | Nguyen et al., 2019 |
| Dexamethasone, betamethasone, darunavir, and lopinavir | P-gp | Maternal-fetal PK | Nonpregnant (control) and pregnant population | 0.8–1.25 | Simcyp | Anoshchenko et al., 2021 |
| Digoxin | OATP4C1 | Moderate and severe renal impairment | Renal impaired population | 0.5–2 | Simcyp | Scotcher et al., 2017 |
| Cidofovir, oseltamivir, carboxylate and cefuroxime | OAT | Severe renal impairment | Renal impaired population | 0.5–2 | Simcyp | Hsu et al., 2014 |
| Adefovir, avibactam, entecavir, famotidine, ganciclovir, oseltamivir, carboxylate and sitagliptin | OAT | Moderate and severe renal impairment | Renal impaired population | 0.67–1.5 | Simcyp | Hsueh et al., 2018 |

*Range (multiple doses and route of administration were tested).

TABLE 5
Interspecies differences in DMET abundance (pmol/mg protein)

| Tissue | DMET Proteins | Human | Monkey | Dog | Rat | Mouse | References |
|---|---|---|---|---|---|---|---|
| Liver | MDR1/Mdr1a | 0.40 ± 0.20 | 0.46 ± 0.10 | 0.59 ± 0.11 | 0.26 ± 0.03 | — | Wang et al., 2015 |
| | | 0.12 ± 0.01 | | | 0.02 ± 0.04 | 0.01 ± 0 | Hammer et al., 2021 |
| | MATE1/Mate1 | 0.48 ± 0.15 | — | — | 0.72 ± 0.15 | — | Wang et al., 2015 |
| | MRP2/Mrp2 | 1.54 ± 0.64 | 5.15 ± 1.53 | 1.93 ± 0.19 | 7.33 ± 1.16 | — | Yin et al., 2015 |
| | | | | | 2.84 ± 0.44 | | Hammer et al., 2021 |
| | MRP3/Mrp3 | 0.51 ± 0.15 | 0.76 ± 0.21 | 0.38 ± 0.05 | BLQ | — | Wang et al., 2015 |
| | | | | | 0.08 ± 0.01 | 0.13 ± 0.06 | Hammer et al., 2021 |
| | MRP4/Mrp4 | BLQ | BLQ | BLQ | BLQ | — | |
| | OCT1/Oct1 | 4.45 ± 1.89 | 18.8 ± 6.8 | 0.50 ± 0.30 | 2.14 ± 0.47 | — | Wang et al., 2015 |
| | BSEP/Bsep | 2.11 ± 0.50 | 4.45 ± 1.48 | 1.87 ± 0.37 | 2.31 ± 0.45 | — | |
| | | 0.23 ± 0.01 | | | 1.02 ± 0.16 | 0.94 ± 0.26 | Hammer et al., 2021 |
| | NTCP/Ntcp | 2.17 ± 0.93 | 1.90 ± 0.24 | — | 7.91 ± 1.36 | — | Wang et al., 2015 |
| | | 0.21 ± 0.03 | | | 1.74 ± 0.23 | 19.21 ± 3.52 | Hammer et al., 2021 |
| | Oatp1a2 | | | 0.70 ± 0.17 | | | |
| | Oatp1b4 | | | 10.4 ± 2.3 | | | Wang et al., 2015 |
| | OATP2B1/Oatp2b1 | 1.7 ± 0.6 | 0.30 ± 0.10 | 4.55 ± 2.00 | | | |
| | OATP1B1/Oatp1b1 | 2.0 ± 0.9 | 12.7 ± 1.4 | | | | |
| | OATP1B3/Oatp1b3 | 1.1 ± 0.5 | 14.4 ± 2.1 | | | | |
| | Oatp1a1 | | | | 3.91 ± 0.84 | | |
| | Oatp1a4 | | | | 5.56 ± 1.40 | | |
| | Oatp1b2 | | | | 6.43 ± 1.40 | | |
| | CYP1A1/Cyp1a1 | 0.09 ± 0.01 | | | 0.05 ± 0 | | |
| | CYP1A2/Cyp1a2 | 5.93 ± 0.45 | | | 1.3 ± 0.49 | | Hammer et al., 2021 |
| | CYP2B6 | 0.59 ± 0.04 | | | | | |
| | CYP2C8 | 7.01 ± 0.55 | | | | | |
| | CYP2C9 | 14.87 ± 0.67 | | | | | |
| | CYP2C19 | 2.29 ± 0.07 | | | | | |
| | CYP2D6 | 4.35 ± 0.25 | | | | | |
| | CYP2E1 | 3.81 ± 0.2 | | | | | |
| | CYP3A4 | 4.8 ± 0.2 | | | | | |
| | CYP3A5 | 0.77 ± 0.04 | | | | | |
| | Cyp2b10 | | | | | 0.21 ± 0 | |
| | Cyp2c29 | | | | | 8.07 ± 0.93 | |
| | Cyp2c38 | | | | | 0.03 ± 0 | |
| | Cyp2c39 | | | | | 0 ± 0 | |
| | Cyp2c55 | | | | 0.01 ± 0 | 0.18 ± 0.03 | |
| | Cyp2d9 | | | | | 18.4 ± 1.8 | |
| | Cyp2d10 | | | | | 16.7 ± 2.0 | |
| | Cyp2e1 | | | | 5.86 ± 0.97 | 32.8 ± 2.2 | |
| | Cyp3a25 | | | | | 0.24 ± 0.09 | |
| | Cyp2b1 | | | | 0.1 ± 0.03 | | |
| | Cyp2c11 | | | | 57.8 ± 15.7 | | |
| | Cyp2c12 | | | | 0.43 ± 0.41 | | |
| | Cyp2c13 | | | | 2.7 ± 2.7 | | |
| | Cyp2d3 | | | | 11.1 ± 2.2 | | |
| | Cyp3a9 | | | | 0.48 ± 0.15 | | |
| | Cyp3a18 | | | | 1.0 ± 0.4 | | |
| | AO | 11.96 | 17.87 | | | | Basit et al., 2021 |
| | CES1 | 569.06 | 153.55 | | | | |
| | CES2 | 64.85 | 491.69 | | | | |
| | SULT1A1 | 5.97 | 23.01 | | | | |
| | SULT1B1 | 0.23 | 0.31 | | | | |
| | SULT1E1 | 1.07 | 1.60 | | | | |
| | SULT2A1 | 18.59 | 17.63 | | | | |
| | SULT1A3 | 1.98 | | | | | |
| | UGT1A1 | 0.87 | 0.29 | | | | |
| | UGT1A3 | 0.3 | 0.28 | | | | |
| | UGT1A6 | 1.02 | 5.70 | | | | |
| | UGT2B4 | 5.83 | 32.25 | | | | |
| | UGT2B7 | 6.01 | 20.73 | | | | |
| | UGT2B15 | 3.76 | 7.92 | | | | |
| | UGT2B17 | 0.21 | | | | | |
| Kidney | MDR1/Mdr1a | 2.11 ± 0.68 | 1.86 ± 0.34 | 1.08 ± 0.35 | 1.13 ± 0.28 | 0.56 ± 0.21 | |
| | MATE1/Mate1 | 5.21 ± 2.11 | 5.76 ± 1.75 | — | — | — | Basit et al., 2019 |
| | MRP4/Mrp4 | 0.91 ± 0.62 | 2.58 ± 0.74 | — | 1.09 ± 0.21 | 0.32 ± 0.14 | |
| | OAT1/Oat1 | 5.21 ± 1.82 | 8.51 ± 1.59 | 2.37 ± 0.94 | 8.96 ± 1.97 | 5.19 ± 3.25 | |
| | OAT2/Oat2 | 0.97 ± 0.32 | 0.96 ± 0.3 | — | BLQ | 4.12 ± 1.37 | |
| | OAT3/Oat3 | 3.84 ± 1.3 | 4.44 ± 1.03 | — | — | — | |
| | OAT4/Oat4 | 0.51 ± 0.23 | 0.62 ± 0.2 | — | — | — | |
| | OCT2/Oct2 | 8.16 ± 2.34 | 16.72 ± 4.84 | | 7.32 ± 1.76 | 14.7 ± 4.88 | |
| | OCTN1/Octn1 | 1.32 ± 0.51 | 0.63 ± 0.3 | — | — | — | |
| | OCTN2/Octn2 | 0.64 ± 0.2 | 1.55 ± 0.33 | 0.53 ± 0.18 | 2.8 ± 0.59 | 1.48 ± 0.31 | |
| | SGLT2/sglt2 | 3.68 ± 1.51 | 6.39 ± 1.34 | 11.89 ± 2.87 | 11.6 ± 2.25 | 13.66 ± 2.91 | |
| | AO | 1.53 | 3.10 | | | | Basit et al., 2021 |
| | CES1 | 3.65 | 44.14 | | | | |

(*continued*)

TABLE 5—*Continued*

| Tissue | DMET Proteins | Human | Monkey | Dog | Rat | Mouse | References |
|---|---|---|---|---|---|---|---|
| | CES2 | 35.1 | 72.48 | | | | |
| | SULT1A1 | BLQ | 2.97 | | | | |
| | SULT1B1 | BLQ | 0.15 | | | | |
| | SULT1E1 | BLQ | 1.05 | | | | |
| | SULT2A1 | BLQ | 0.40 | | | | |
| | SULT1A3 | BLQ | | | | | |
| | UGT1A1 | BLQ | 0.06 | | | | |
| | UGT1A3 | BLQ | 0.04 | | | | |
| | UGT1A6 | 0.22 | 0.41 | | | | |
| | UGT2B4 | BLQ | 0.23 | | | | |
| | UGT2B7 | 1.74 | 2.46 | | | | |
| | UGT2B15 | BLQ | 1.17 | | | | |
| Intestine | AO | BLQ | 1.25 | | | | Basit et al., 2021 |
| | CES1 | 3.5 | 62.37 | | | | |
| | CES2 | 224.34 | 388.08 | | | | |
| | SULT1A1 | 3.44 | 15.18 | | | | |
| | SULT1B1 | 1.74 | 1.25 | | | | |
| | SULT1E1 | 1.08 | 5.29 | | | | |
| | SULT2A1 | 4.88 | 7.56 | | | | |
| | SULT1A3 | 6.8 | | | | | |
| | UGT1A1 | 0.97 | 4.32 | | | | |
| | UGT1A3 | 0.03 | 4.58 | | | | |
| | UGT1A6 | BLQ | 0.88 | | | | |
| | UGT2B4 | BLQ | 0.12 | | | | |
| | UGT2B7 | 1.88 | 0.83 | | | | |
| | UGT2B15 | | 2.23 | | | | |
| | UGT2B17 | 1.31 | | | | | |

(Yu et al., 2006) as well as for the quantification of plasma membrane transporters expressed in CHO and MDCK cells (Kumar et al., 2017b).

### *D. Technical Challenges and Quality Controls*

Rigor and reproducibility are important attributes of a quantitative method. The selection of optimum quality controls provides confidence in the results by minimizing technical variability in data. Interlaboratory variability in protein quantification is common in the absence of harmonized guidelines for quantitative proteomics. The factors contributing to the inter- or within-laboratory variability in protein quantification typically include the use of different matrices (e.g., homogenate versus cell fractions), different digestion protocols [e.g., filter-assisted sample preparation (FASP) versus immunoenrichment], different measurement techniques (targeted versus untargeted), variability in sample extraction efficiency, and inadequately optimized proteotypic peptides. Therefore, the use of internal and external quality controls (discussed below) is required for minimizing the technical variability in protein quantification results.

*1. Multiple Proteotypic Peptides and Multiple Product Ions.* The use of multiple proteotypic peptides of a protein provides confidence in protein quantification by eliminating bias caused by changes in amino acid due to SNP, PTM, and peptide stability. A good correlation between the responses of multiple peptides across samples can be used to rule out peptide-specific variability. Similarly, the selection of multiple product ions of a peptide should be measured for reproducible protein quantification. A strong correlation (generally $r^2 > 0.99$) between the responses of the selected product ions confirms the absence of any background interferences.

*2. Reanalysis of Incurred Samples.* Reanalysis of a sample with known concentrations of proteins of interest [also referred to as positive quality control (PQC) sample] assists in evaluating the batch-to-batch variability. Data normalization by the PQC sample minimizes intra- and interday variations in the sample preparation and analysis steps. Further, the use of a universal PQC sample for data normalization can be implemented to reduce interlaboratory variability.

*3. Peptide and Protein Internal Standards.* Externally added SIL peptides as an internal standard address the variations due to postdigestion factors such as the matrix effect, MS instrument sensitivity, retention time shift, and sample evaporation. Similarly, the spiking of an exogenously added protein (e.g., bovine serum albumin) during the digestion can serve as an internal control for the predigestion, sample processing, and instrumentation variability.

*4. Data Normalization by Marker Proteins.* Subcellular fractions (e.g., tissue homogenate, S9 fractions, cytosol, and microsomes) used for in vitro drug metabolism and transporter studies are susceptible to interbatch or interlaboratory variability, which often leads to a poor IVIVE. For example, to extrapolate enzyme-mediated drug clearance from subcellular fraction to a tissue or organ clearance, universal scaling factors [e.g., a milligram of protein per gram of liver tissue (MPPGL)] are required. Identification of marker (housekeeping) proteins (Supplemental Table 4) can be used to calculate recovery, enrichment, and purity in the in vitro preparations (Xu et al., 2018). The marker proteins can also be utilized for characterizing the

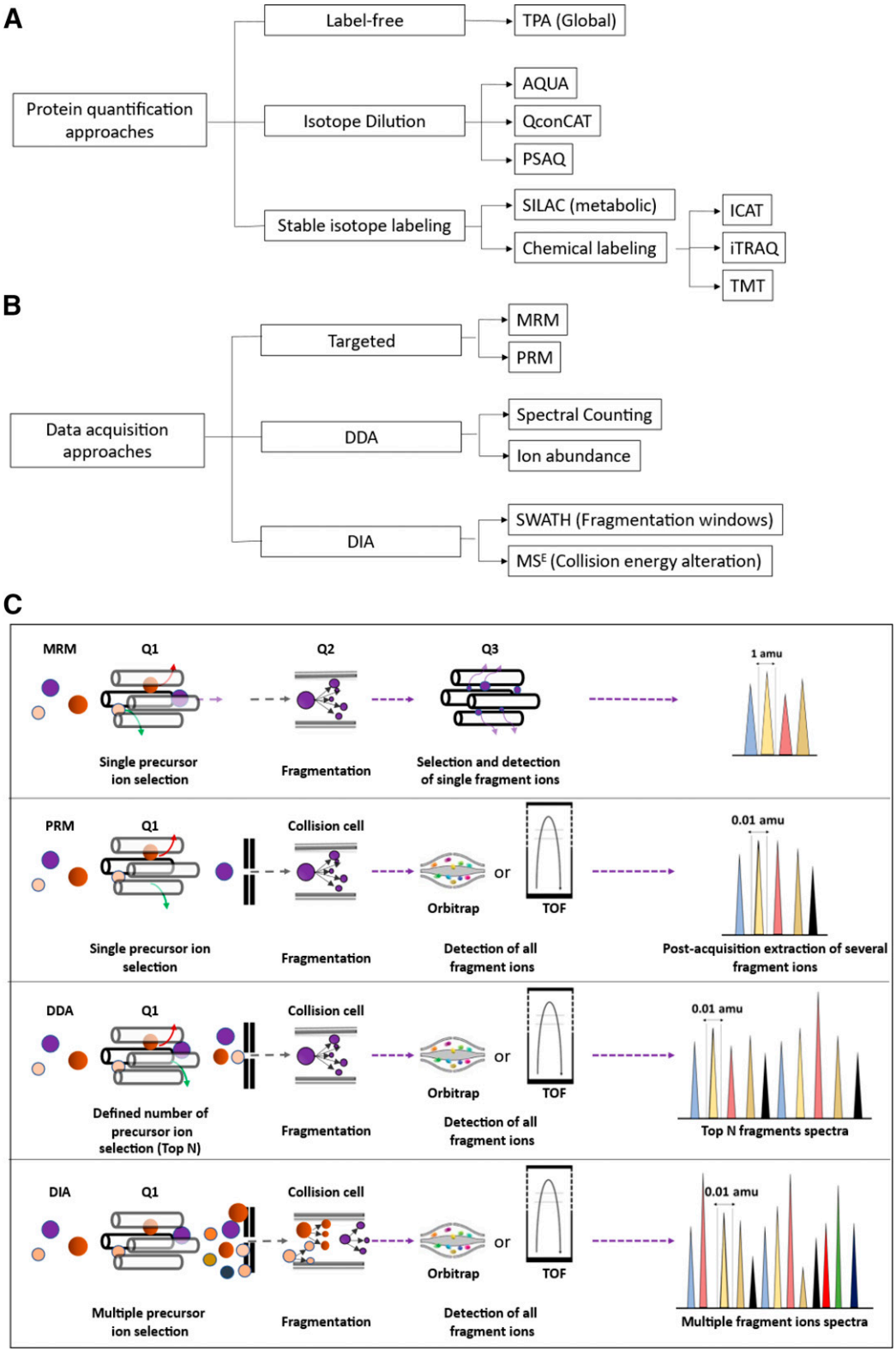


**Fig. 1.** Absolute and relative LC-MS/MS–based protein quantification (A) and proteomics data acquisition (B) approaches. (C) Schematic depiction of various mass-spectrometry data-acquisition approaches for targeted and untargeted proteomics. MRM can be performed using low-resolution MS analyzers such as triple quadrupole and Q-trap, but PRM, DDA, and DIA require high-resolution MS analyzers such as orbitrap or TOF. AQUA, absolute quantification.

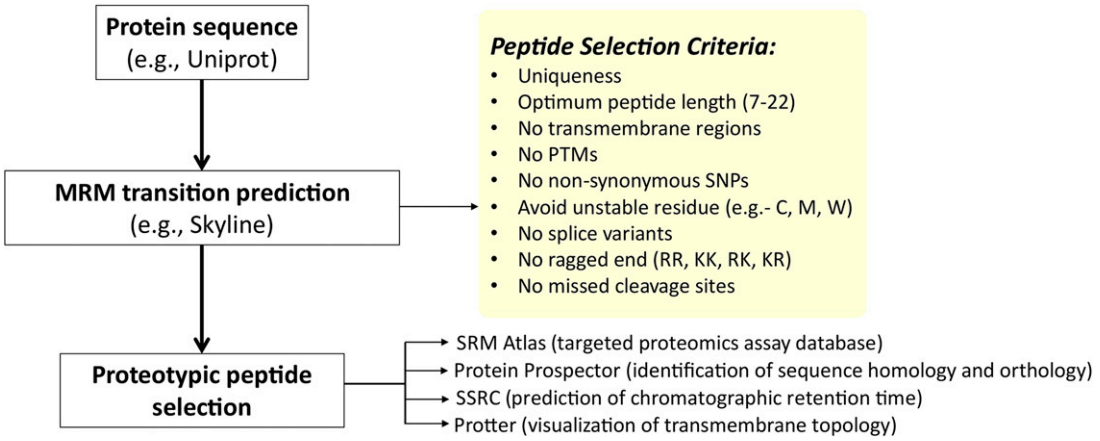


**Fig. 2.** Steps involved in proteotypic peptide selection for the targeted quantitative proteomics. First, protein sequence is acquired from databases such as Uniprot (https://www.uniprot.org/). MRM transitions are predicted using Skyline (https://skyline.ms/project/home/begin.view?). Finally, proteotypic peptides can be selected based on criteria listed on the top-right corner using a series of software tools such as SRM Atlas (https://db.systemsbiology.net/sbeams/cgi/PeptideAtlas/GetTransitions), Protein Prospector (https://prospector.ucsf.edu/prospector/cgi-bin/msform.cgi?form=mshomology), sequence-specific retention calculator (SSRC) (http://hs2.proteome.ca/SSRCalc/SSRCalcx.html), or Protter tool within Skyline. SRM, Selected Reaction Monitoring.

in vitro subcellular fraction that is critical for IVIVE. Similarly, the marker proteins can identify variability in tissue sampling in heterogeneous tissue, e.g., kidney (Li et al., 2019b).

*5. Identifying Sample Integrity Issues.* Tissue samples are particularly susceptible to quality issues. Principal component analysis (PCA) is a dimensionless method that reduces the complexity of a large number of variables by transforming them into smaller ones (principal components, e.g., PC1, PC2, etc.). Since smaller data sets are easy to visualize and analyze, the overall variability can be interpreted by machine learning algorithms in PCA (Rao and Li, 2009). Thus, PCA can be used not only to interpret data for changes caused by biological factors but also in identifying poor quality samples in a big sample cohort (Yan et al., 2008; Rao and Li, 2009; Bhatt and Prasad, 2018).

## III. Applications of Quantitative Proteomics in Translational Absorption, Distribution, Metabolism, and Excretion and Precision Medicine

### *A. Characterization of In Vitro Models*

In vitro models are crucial for ADME screening because of their ability to recapitulate human mechanisms, generate data in a cost-effective and high-throughput manner, avoid cross-species differences, and minimize animal use. Human liver microsomes, cytosol, hepatocytes, recombinant proteins, and transporter-expressing cell lines or vesicles are commonly used for in vitro screening of drug metabolism and transport as well as for predicting in vivo clearance and DDI potential. However, the in vitro models suffer from several limitations e.g., (1) pooled human microsomes or cytosol do not represent the population variability, (2) DMET abundance or activity changes with cell culture conditions or between lots, and (3) DMET abundance in recombinant enzyme systems or transporter-overexpressing cell lines and vesicles is not similar to corresponding human tissue. To address these limitations, quantitative proteomics has been used to assess the effect of cell culture conditions on transporter abundance in human hepatocytes in relation to the freshly isolated or cryopreserved human hepatocytes (Kumar et al., 2019). Similarly, quantitative proteomics allows the determination of the relative expression factor (REF) values between the recombinant models and human tissue for tissue-specific extrapolation of the intrinsic clearance data (Parvez et al., 2021).

Microsomal enrichment and recovery are influenced by the processing variables such as the choice of homogenizer, buffer composition, and centrifugation speed resulting in technical variability in the enzyme abundance or activity (Xu et al., 2018). Interestingly, the impact of microsomal or cytosolic processing variables is not consistent for different enzymes as it depends on the subcellular localization of proteins. For example, carboxylesterases (CESs) are soluble endoplasmic reticulum luminal proteins, which are susceptible to release into cytosolic fraction during the microsome preparation. Although microsomal fraction is used for CES activity, it does not account for the loss into the cytosolic fraction during the microsomal preparation. Plasma membrane isolation for transporter quantification suffers from similar challenges of contamination from other subcellular organelles (Kumar et al., 2015). Multiplexed quantification of subcellular markers (Supplemental Table 4) by quantitative proteomics is capable of assessing the purity of microsomal, cytosolic, and membrane preparations (Xu et al., 2018).

Overexpressed cell lines (MDCK or LLC-PK1 cells) are commonly used in vitro models to characterize transporter substrate or inhibitor potential of new drugs. However, large interlaboratory differences in transporter expression across laboratories reduce confidence in translating these data to humans. Quantitative proteomics has been used to delineate differences between the overexpressed in vitro models and tissue preparations (Table 1). Breast cancer resistance protein (BCRP/ABCG2) and bile salt export pump (BSEP/ABCG11) were quantified in overexpressing insect membrane vesicles and rat, dog, monkey, and human liver samples for the extrapolation of PK parameters of biliary excretion from in vitro models to humans (Li et al., 2009). Similarly, systemic characterization of four different P-glycoprotein (P-gp)-overexpressing cell lines for the abundance resolved IVIVE disconnect in another study (Li et al., 2021).

Because of the high cost and logistical challenges of obtaining good quality human tissues and primary cells, cell lines such as Caco-2, HepG2, HepaRG, HUH7, and LS180 cells are also used in ADME assays. Characterization of the ability of these models to metabolize or transport drugs, including the effect of culture conditions on the DMET abundance, is important for better translation of the in vitro data (Table 1). Recently, advances in cell culture and microfluidics techniques have led to the development of microphysiologic systems (MPS) that are proving to be promising in drug screening. However, accurate determination of DMET proteins in MPS models is essential for the validation of these tools. Proteomic characterization in MPS models is often challenging due to low initial cell counts (5000–20 000 cells), especially using the modest sensitivity of conventional LC-MS instruments. Advances in LC-MS/MS sensitivity and miniaturized sample preparation are expected to address this limitation.

### *B. In Vitro to In Vivo Extrapolation*

Although in vitro models play crucial roles in the early screening of undesirable characteristics of an NCE, accurate scaling of in vitro data to humans is critical for safer and cost-effective drug development. In vitro metabolic or transporter-mediated clearance data are used to predict in vivo clearance, DDI, as well as in vivo human PK to estimate the first-in-human dose. However, the lack of knowledge concerning physiologic differences, including DMET abundance between in vitro models and human tissues, often leads to the poor scaling of in vitro data. The relative activity factor (RAF) approach (eq. 1) that relies on differential activity measured using a probe substrate between recombinant system and human tissue is used for IVIVE of drug intrinsic clearance. However, specific probe substrates are not available for the majority of DMET proteins. Moreover, this approach is not viable for transporter assays as the uptake and efflux activity cannot be measured in tissue samples.

RAF Approach

$$CL_{int_{tissue}} = CL_{int_{recombinant}} \times \frac{[CL_{int}\text{probe substrate in HLM}]\left(\frac{pmol/\text{min}}{mg}\right)}{[CL_{int}\text{probe substrate in } recombinant\ enzyme]\left(\frac{pmol/\text{min}}{mg}\right)} \times MPPGL\,(mg/gram) \times organ\ weight\,(gm) \quad (1)$$

REF Approach

$$CL_{\text{int}_{HLM}} = CL_{\text{int}_{recombinant}} \times \frac{[\text{Enzyme abundance}_{\text{HLM}}]\left(\frac{pmol}{mg}\right)}{[\text{Enzyme abundance}_{recombinant}]\left(\frac{pmol}{mg}\right)} \times \text{MPPGL}\,(\text{mg/gram}) \times \text{organ weight}\,(\text{gm}) \quad (2)$$

Proteomics-informed IVIVE utilizing the REF approach (eq. 2) is an emerging alternative to address the limitations of the RAF approach. Typically, the enzyme or transport kinetics data in the recombinant system provide an estimation of in vitro velocity maximum ($V_{max}$) and substrate affinity ($K_m$) values to derive intrinsic clearance ($CL_{int}$). Since $V_{max}$ is proportional to the protein expression, and $K_m$ is considered constant between systems (tissue versus in vitro system), the REF approach can be used to normalize the kinetics data by protein abundance in tissues to estimate tissue-intrinsic clearance by individual enzymes (eq. 2). The DMET abundance–normalized $CL_{int}$ data can be extrapolated to organ clearance by multiplying it with two systems scaling factors, e.g., MPPGL or cytosolic protein per gram of liver (CPPGL) and organ weight (eqs. 1 and 2). Proteomics-informed scaling of in vitro transport data from OCT1-transporter–expressing HEK293 cells and plated human hepatocytes resulted in a reasonable prediction of in vivo clearance (Sachar et al., 2020). Likewise, IVIVE of transporter-mediated renal clearance of 26 OAT substrates by proteomics-based REF approach was able to predict the renal clearance within 2- to 3-fold of the observed values (Kumar et al., 2021). Quantitative proteomics data can also be used to predict tissue-specific toxicity by integrating DMET abundance and in vitro metabolism and transporter data. For example, selective intestinal toxicity of SN-38 can be explained by the higher abundance of CES2 relative to UGT1A1 in the intestine in comparison with the liver (Parvez et al., 2021). Further, quantitative proteomics analysis of microbial gut β-glucuronidases showed a good correlation with higher intestinal toxicity of SN38 as the former facilitates the conversion of SN38-glucuronide to the toxic SN38 (Parvez et al., 2021). DMET proteins are expressed in various tissues besides the liver. Quantification of DMET proteins in different tissues can be used to determine tissue-specific $f_m$ or $f_t$ to tease out the effect of DDIs and pharmacogenetic variability

on local tissue concentration and toxicity. For example, MK-7246, a selective CRTH2 prostaglandin 2 antagonist, was discontinued from clinical development due to high variability in PK (Wang et al., 2012). Although MK-7246 is metabolized by multiple UGTs in the liver, retrospective pharmacogenetic data suggested 96% metabolism by UGT2B17 ($f_m$ = 0.96), which could only be explained by the high intestinal abundance of UGT2B17 resulting in the high first-pass metabolism of the investigational drug (Wang et al., 2012). Similarly, proteomics data were important for explaining preferential MRP2-mediated biliary secretion of androgen glucuronides of testosterone and other androgens. Although vesicle data indicated the predominant role of sinusoidal MRP3 in the excretion of androgen glucuronides into the blood, the higher abundance of canalicular MRP2 suggested a preferential elimination of androgen glucuronides into the intestinal lumen and explained the enterohepatic recycling (Li et al., 2019a). Quantitative proteomics has also been used to assess the impact of genetic variability on clinical outcomes. For instance, the interindividual variability in the in vitro metabolism of (S)-mephenytoin and clopidogrel catalyzed by CYP2C19 in the human liver was successfully predicted by the changes in CYP2C19 protein abundance (Shirasaka et al., 2016).

Primary human hepatocyte culture is considered the gold standard for the evaluation of enzyme induction potential of new chemical entities, which relies on specific substrates for the activity assays. However, the selective probe substrates are often not available, especially for the highly homologous proteins, such as CYP3A4, CYP3A5, and CYP3A7. Although mRNA expression could predict changes in transcription, it often does not provide quantitative changes in protein abundance. Quantitative proteomics serves as a better alternative to determine the enzyme or transporter induction potential because it offers multiplex protein quantification with greater throughput, sensitivity, and robustness as compared with the currently recommended methods (mRNA and activity assay) by the regulatory agencies. Quantitative proteomics was used for the accurate determination of the induction potential of CYP1A2, CYP2B6, CYP3A4, and CYP3A5 in human hepatocyte incubations (Williamson et al., 2011). Similarly, immunoaffinity-based proteomic approach was used in another study for the assessment of enzyme induction potential in a small sample amount (MacLean et al., 2017). Recent data further validate the use of quantitative proteomics for assessing the induction of the enzyme protein abundance in human hepatocytes, which reasonably precited the in vitro enzyme activity data using probe substrates (Stresser et al., 2021; Savaryn et al., 2022). Because protein quantification better reflects the functional activity as compared with mRNA, multiplexed DMET quantification by quantitative proteomics has the potential to screen the protein induction and suppression potential during routine early drug discovery stages.

### C. Prediction of Interindividual Variability

Drug ADME and response is influenced by several population factors such as age, sex, genotype, disease conditions, and other health conditions like pregnancy. However, due to ethical, logistical, and financial reasons, clinical trials are typically performed on a limited number of adult subjects. With the emergence of PBPK modeling, potential interindividual variability in drug metabolism and PK can be predicted using virtual populations as far as the physiologic data like DMET protein abundance in different populations are known. Table 2 provides a compilation of the quantitative proteomics studies used for predicting interindividual variability in drug disposition. The principle of drug clearance translation from healthy adults to a special population, such as pediatric, depends on the fact that most of the extrinsic and intrinsic factors (Fig. 3) affect protein abundance or $V_{max}$ without influencing $K_m$. Therefore, characterization of the influence of population factors on DMET abundance is critical information for a successful prediction of the interindividual variability using PBPK modeling.

*1. Effect of Age.* It is well recognized that children are not small adults. Age is associated with multifactorial physiological changes, especially during early life, and the abundance of different DMET proteins changes with age in a nonmonotonic fashion, resulting in variable drug clearance and unpredictable drug toxicity or efficacy. For example, the underdevelopment of UGT enzymes has been shown to correlate with the immature glucuronidation activity in neonates and infants, which could lead to toxicity of UGT substrates when prescribed based on body weight or body surface area–normalized doses. Chloramphenicol caused cardiovascular collapse characterized by Gray baby syndrome in neonates due to the poor glucuronidation capacity of younger children (Mulhall et al., 1983). Similarly, lower levels of UGTs are associated with higher exposure of zidovudine in neonates and infants, leading to hematological toxicity (Capparelli et al., 2003). Further, age-dependent maturation of DMET abundance could confound the effect of genetic variability in the case of highly polymorphic enzymes or transporters (e.g., CYP2D6, UGT2B17, OATP1B1, and OCT1). The well-documented adverse effects of codeine in breastfed infants can be explained by extensive maternal CYP2D6 activity as well as the low abundance of the detoxifying enzyme UGT2B7 in infants (Bhatt et al., 2019).

Age-dependent changes in DMET quantitative proteomics data in the human liver are compiled in Table 2 and Fig. 4. The developmental patterns of DMET proteins are generally characterized into three groups. Some proteins show the highest

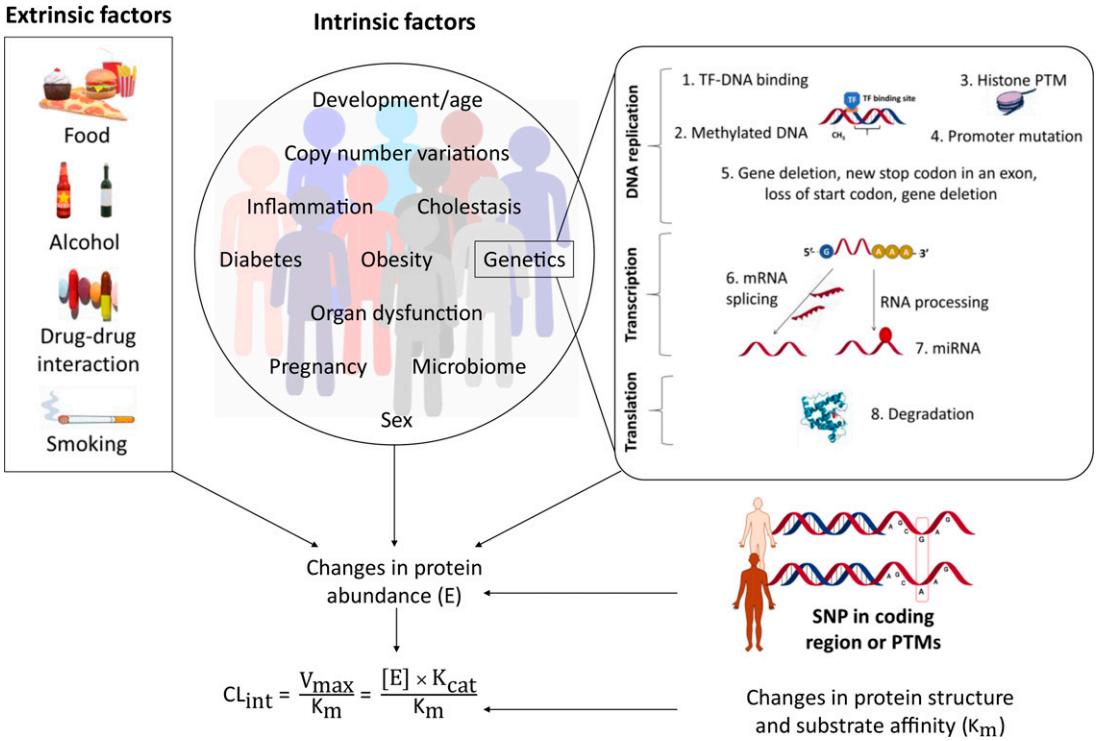


**Fig. 3.** Effect of internal and extremal factors on $CL_{int}$ of drugs via changes in DMET protein abundance or substrate affinity. Most of these factors affect the velocity maximum ($V_{max}$) by altering protein abundance (E), whereas substrate affinity ($K_m$) can be affected by changes in amino acid sequences at active site by factors such as PTMs or nonsynonymous SNPs affecting active site. $K_{cat}$, turnover number; miRNA, microRNA.

abundance in neonates which decreases with age. For example, CYP3A7 sharply decreases during the first year of life and is rarely detected in adults. Similarly, BCRP is decreased with age in the human liver, but the change in its abundance remains within ~2-fold between neonates and adults. The second group of DMET proteins is relatively constant (<1.5-fold change) throughout the development. Examples of these proteins include CYP2C9, CYP2E1, CYP3A5, SULT1A1, SULT2A1, OATP1B1, OATP2B1, NTCP, BSEP, and MATE1. However, the majority of DMET proteins belong to the third group, which mature with age (group 3). The group 3 proteins include CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP3A4, UGT1A1, UGT1A4, UGT1A6, UGT1A9, UGT2B7, UGT2B15, UGT2B17, ADH1A, ADH1B, and ADH1C and show a >5-fold difference in the abundance from neonatal stage to adulthood. These ontogeny data, which are now routinely derived using quantitative proteomics, have been integrated into PBPK software such as Gastroplus and Simcyp for the extrapolation of adult PK data to children. Since the regulatory agencies are encouraging clinical trials in children (https://www.nichd.nih.gov/research/supported/bpca), protein abundance–informed pediatric PBPK modeling is important for designing safer clinical studies in children.

*2. Effect of Genetic and Epigenetic Factors.* Genetic polymorphisms in DMET proteins contributes significantly to the PK variability of drugs. In general, there are approximately 14–16 million SNPs that are observed every 300–1000 nucleotides across the human genome (Roden and George, 2002). The $V_{max}$ of metabolism/transporter is affected by a direct effect on the protein abundance as a result of changes such as a mutation in transcription factor–binding site or promotor region, insertion of a new stop codon in an exon, gene deletion, loss of start codon, mRNA splicing, or protein degradation (Fig. 3), although the nonsynonyms SNPs can also affect $K_m$. The influence of genetic polymorphism on drug PK may require dosing adjustment for certain individuals, especially for the narrow therapeutic index drugs (Collins, 1991). For example, genetic variations in patients have been shown to be associated with unpredictable toxicity or the lack of efficacy (Shuldiner et al., 2009; Avivi et al., 2014). Table 2 presents representative examples of clinically relevant SNPs, copy number variation (CNV), and epigenetic changes that are commonly associated with population variability in drug PK and/or response.

Genetic polymorphisms that are caused by variations at a SNP or haplotype in a gene of a DMET protein are one of the most common causes of interindividual variability in PK. For example, CYP2D6 exhibits more than

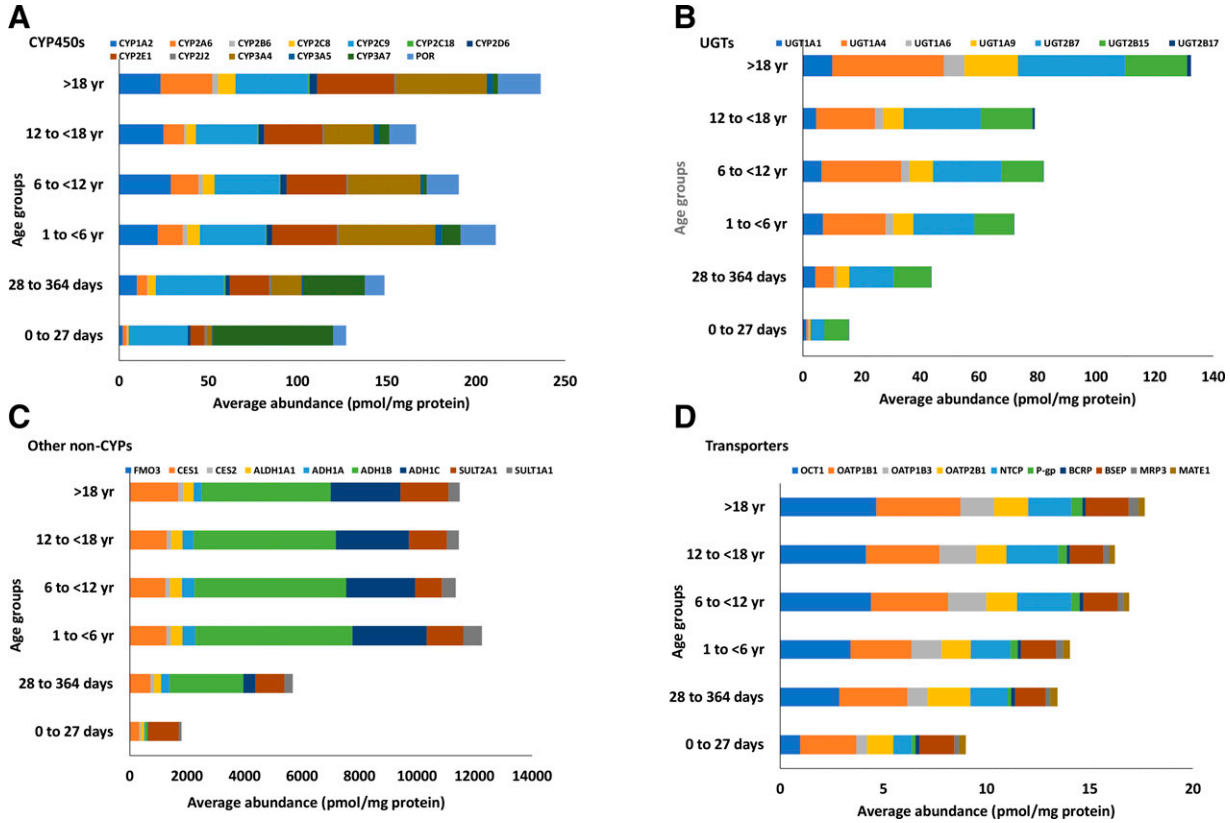


**Fig. 4.** Age-dependent changes in the abundance of CYPs (A), UGTs (B), transporters (C), and other non-CYPs (D) in the liver from human donors representing neonatal, infants, early-childhood, middle-childhood, adolescents, and adults.

100 genetic variants due to point mutations, duplications, and insertion or deletion of single or multiple nucleotides. Individuals with different CYP2D6 allelic variants are classified as poor metabolizers (PMs), intermediate metabolizers (IMs), extensive metabolizers (EMs), and ultrarapid metabolizers (UMs), corresponding to the extent of involvement of these variants in drug metabolism (Zhou et al., 2008). For example, tamoxifen, a selective estrogen receptor (ER) modulator used in $ER^+$ breast cancer patients, is extensively metabolized by CYP2D6 to endoxifen (Filipski et al., 2014). Due to the genetic variability in CYP2D6, ultrarapid metabolizers experience a higher plasma level of endoxifen than poor metabolizer and intermediate metabolizer patients and encounter severe hot flashes and a higher risk of disease relapse (Goetz et al., 2005). In addition, SNPs in the coding region of an enzyme or transporter could change the $K_m$ without changing the protein abundance. For example, CYP2D6*17 allele, which carries T107I, R296C, and S486T mutations, leads to a reduced function of CYP2D6 by increasing the $K_m$ (decreased affinity) for bufuralol and dextromethorphan (Marcucci et al., 2002).

CNV is a structural variation in which a section of the gene is repeated or deleted (~1 kb to 3 Mb). CNVs are reported for several DMEs such as CYP2A6, CYP2C19, CYP2D6, CYP3A5, UGT2B17, GSTM1, GSTT1, and SULT1A1 (Ménard et al., 2009; Zhou et al., 2010; Santos et al., 2018). Interestingly, UGT2B17 shows a very high ethnic variability in the frequency of individuals with the gene deletion (*2/*2) (Xue et al., 2008), i.e., ~25% in the African and European populations but >70% in the East Asian population (e.g., >90% in the Japanese population). Not only CNV, but several SNPs (rs9996186, rs4860305, and rs28374627) are also associated with UGT2B17 abundance (Bhatt et al., 2017). Similarly, the changes in CYP2C19 abundance due to genetic polymorphisms strongly correlated ($r^2$ = 0.984) with (S)-mephenytoin hydroxylase activity (Fig. 5A) (Shirasaka et al., 2016). The activity score of CYP2D6 also showed a reasonable correlation with the protein abundance (Fig. 5B) (Gaedigk et al., 2018). Several other proteins such as OATP1B1, BCRP, MRP2, and FMO3 show a strong association of genetic polymorphism with protein abundance that correlates with the in vivo activity (Table 3). Like the expression quantitative trait loci (eQTLs) for the association of genotype and transcript levels, the protein abundance quantitative trait loci (pQTLs) can be used as a noninvasive approach to assess the effect of pharmacogenomic variability. The epigenetic modifications such as DNA methylation, histone modification, and microRNA (miRNA) also contribute to interindividual variability in drug PK due to their effects on DMET protein abundance (Kacevska

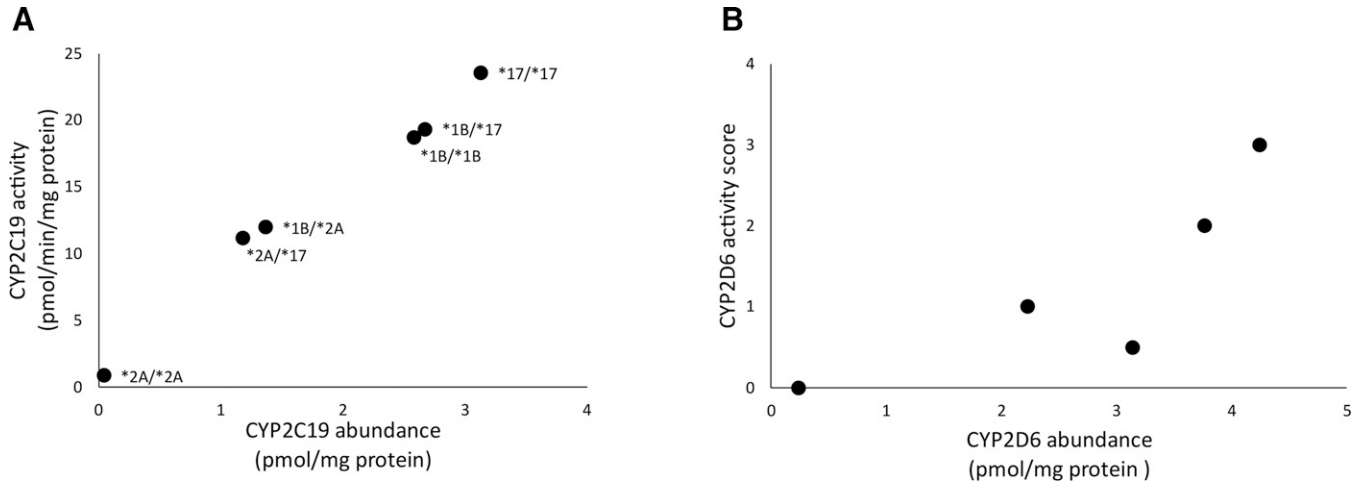


**Fig. 5.** Activity and protein abundance correlation of CYP2C19 (A) and CYP2D6 (B) in human liver microsomes (HLMs) (Shirasaka et al., 2016; Gaedigk et al., 2018).

et al., 2012). For example, miR-491-3p regulates UGT1A3, UGT1A6, and UGT1A1 expression and alters enzyme activity (Dluzen et al., 2014). Thus, integration of quantitative proteomics data into PBPK modeling is an important noninvasive approach to predict the effect of genetic and epigenetic variability on drug PK and response, hence providing a promising approach for precision medicine.

*3. Effect of Sex.* Sex-dependent changes in DMET protein abundance can also contribute to variability in human PK study, drug efficacy, and toxicity. Erythromycin is primarily metabolized by CYP3A4 in the human liver to N-demethylated metabolite, which showed 40% more metabolism in women than men. Other CYP3A4 substrates such as verapamil, nifedipine, mirtazapine, cyclosporine, and diazepam also show higher metabolic clearance in women (Nicolas et al., 2009). Regarding phase 2 metabolism, acetaminophen clearance is 22% higher in men than women due to the higher glucuronidation rate (Miners et al., 1983), whereas aspirin exhibits higher bioavailability in women due to the reduced conjugation by glucuronic acid and glycin (González-Correa et al., 2007). The levels of UGT2B17 in the human liver quantified by quantitative proteomics showed ∼3-fold higher abundance in men (Bhatt et al., 2018). Likewise, BCRP, CYP2A6, and FMO3 showed ∼1- to 2-fold higher abundance in men (Table 2). Although the sex differences in DMET proteins are small in humans, more dramatic sex-dependent changes have been reported in rodents. In particular, quantitative proteomics data revealed the highest sex-dependent changes in kidney transporters in mice, followed by rats and dogs. Kidney OAT1 is ∼3.2- and 1.3-fold higher in male mice and rats, respectively, whereas OAT2 is 1.6-fold higher in male mice than in female mice. Mdr1 is 2- and 1.4-fold higher in the kidneys of female mice and dogs, respectively (Basit et al., 2019). These proteomics data in preclinical species correlate with the in vivo PK and toxicity data (Diamond et al., 2010; Lolkema et al., 2015). Overall, quantitative tissue proteomics data on sex-dependent differences in DMET proteins in both human and preclinical species are emerging to fill this critical knowledge gap.

*4. Effect of Disease States.* Disease states such as cirrhosis, chronic kidney disease (CKD), cancers, gastrointestinal disorders, autoimmune diseases, and diabetes can alter PK by changing blood flow to organs, plasma protein binding, and apparent volume of distribution, as well as the protein abundance of DMET (Sharma et al., 2020). In particular, the changes in DMET protein abundance can lead to a subtherapeutic response, drug toxicity, DDI, and variable PK profile of drugs. However, it is both ethically and logistically challenging to measure the impact of a disease condition on PK/PD and measure such changes in clinical trials in the disease population. For example, mild, moderate, and severe hepatic or renal dysfunction differentially impacts ADME processes. Since biobanks of postmortem tissues from deidentified donors are becoming increasingly available for research purposes, quantitative proteomics is expected to find broader applications in characterizing disease effects on DMET proteins.

Hepatic dysfunction is one of the major diseases in the United States affecting ∼2% adult population (https://www.cdc.gov/nchs/fastats/liver-disease.htm). The causes of hepatic dysfunction are diverse, including chronic alcohol abuse, viral/bacterial infection, fatty liver disease, and drug-induced injury. Liver diseases significantly affect PK of drugs cleared via hepatic metabolism, albeit the change in the PK depends upon disease severity, mechanisms of drug elimination, and alteration in $f_m$ and $f_t$. Understanding the impact of various liver diseases on DMET abundance is important to

predict drug disposition in a patient with liver disease. The quantitative changes in DMET proteins in hepatic dysfunction are summarized in Table 2. In general, the abundance of DMEs is decreased in the disease state, whereas transporter protein abundance is variable (increased or decreased). The Child-Pugh Class C liver damage is associated with an increase in the expression of P-gp and MRP4 by >200% and a decrease in MRP2, NTCP, OCT1, OATP1B1, and OATP2B1 by <25%–46%. Whereas in the alcoholic liver disease, MRP2, NTCP, OAT2, OATP1B1, OATP1B3, and OATP2B1 are downregulated by 26%–76%. Other hepatic conditions such as Hepatitis C, primary biliary cholangitis, primary sclerosing cholangitis, and autoimmune hepatitis are associated with a reduction in BSEP and OATP2B1 and increase in P-gp and MRP4 (Drozdzik et al., 2020). Wang et al. (2016) used a targeted quantitative proteomics approach and observed a reduced transporter protein expression (except MRP3) in alcoholic cirrhosis patients. Similarly, the effect of nonalcoholic fatty liver disease (NAFLD) of DMET protein expression in steatosis and nonalcoholic steatohepatitis (NASH) liver condition revealed the reduced abundance of OATP1B1/1B3/2B1, OAT2, and NTCP in liver diseases, whereas MRP3 expression was elevated (Vildhede et al., 2020).

CKD is associated with changes in drug PK due to its effect on glomerular filtration rate, protein binding, water loading capacity, and changes in transporter abundance and activity. For example, mirabegron area under the plasma concentration-time curve (AUC) is increased by 1.7-fold in renal impairment patients due to the alteration in P-gp abundance (Dickinson et al., 2013). Similarly, pitavastatin (an OATP substrate) showed 1.4-fold elevated AUC in CKD patients (Morgan et al., 2012). Thus, CKD not only impacts renal elimination of drugs but also modulates the nonrenal clearance of drugs. The impairment of drug metabolism and transport in CKD is considered to be an effect of alterations in protein translations and enzyme degradation, reduced hemoprotein biosynthesis, cofactor depletion, and competitive inhibition of DMEs by circulating uremic constituents (Elston et al., 1993; Nolin et al., 2008). Tan et al. reported a 60% reduction in OPTP1B activity, which can modulate the clearance of nonrenally eliminated drugs such as pitavastatin, repaglinide, etc. (Tan et al., 2019). However, limited quantitative proteomics data are available on the effect of CKD on DMET proteins due to the difficulty in obtaining disease tissue samples for the study. Relying on clinical studies to monitor these multifactorial effects is a resource and cost-demanding effort. Therefore, quantitative proteomics of tissue samples coupled with PBPK modeling is being considered as a promising alternate to clinical studies for predicting disease effects (Prasad et al., 2018; Ladumor et al., 2019b; Rowland Yeo et al., 2020; Effinger et al., 2021).

### *D. Physiologically Based Pharmacokinetic Modeling*

The use of PBPK modeling in drug development and regulatory filing is continuously increasing as evident by more than a twofold increase since the year 2013 (Perry et al., 2020). Although the concept of PBPK modeling is not new (Teorell, 1937), there has been tremendous progress in the application of PBPK models in the recent past. A reliable prediction of drug PK using PBPK modeling requires knowledge of quantitative physiologic data, including DMET abundance. Once validated, these models are considered promising in drug development, such as for dose optimization and clinical study design (Wang et al., 2019). Knowledge of the abundance and activity of DMET proteins in tissues such as the liver, kidney, and intestine is critical for accurate prediction of drug PK in healthy and special populations, such as patients with renal or hepatic impairment, children, and pregnant women (Al Feteisi et al., 2015; Prasad et al., 2017). The proteomics information has been integrated into PBPK modeling (Fig. 6) for predicting drug disposition in special populations (Table 4).

Pediatric PBPK modeling accounts for 20% of all Food and Drug Administration submissions (Zhang et al., 2020b). There are multiple examples of drugs (Table 4) with successful applications of DMET ontogeny data in predicting neonatal and infant PK. For example, a proteomics-based PBPK model successfully predicted the metabolic profile of acetaminophen (a substrate of UGTs, SULTs, and CYPs) in various age groups from neonates to adults (Ladumor et al., 2019a). The abundance data of UGT, SULT, and CYP enzymes were incorporated into the PBPK model to predict the age-dependent change in the fraction of acetaminophen metabolized by individual enzymes ($f_m$). Because $f_m$ is directly proportional to the relative enzyme abundance, the nonmonotonic patterns of enzyme maturation with age is associated with the metabolic switching, i.e., a PBPK model predicted acetaminophen $f_{m,\ ratio}$ values (i.e., $f_{m,UGT}/f_{m,SULT}$) of 0.46, 0.56, and 1.71 in neonates, children, and adults, respectively, which was consistent with the observed clinical data. A PBPK model was used to predict morphine PK in newborns (age 0 days) by using age-dependent changes in the organic cation transporters OCT1 and UGT2B7 (Balyan et al., 2017; Bhatt et al., 2019). Similarly, proteomics-based PBPK modeling has been applied to predict the impact of disease conditions on hepatic drug clearance of drugs such as bosentan, olmesartan, repaglinide, zidovudine, morphine, and telmisartan (Li et al., 2015; Prasad et al., 2018).

### *E. Characterization of Interspecies Differences in Drug Metabolism and Transport*

Interspecies differences in physiologic parameters including DMET protein abundance/activity result in

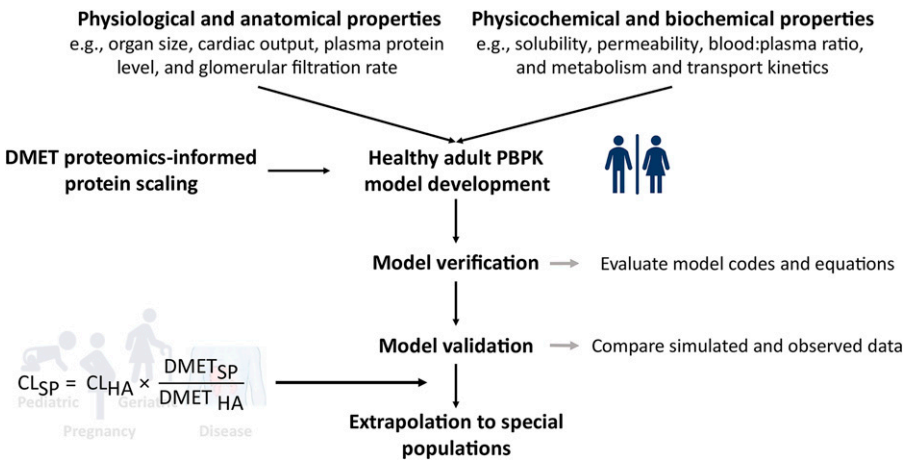


**Fig. 6.** Schematic workflow of proteomics-informed PBPK model development and validation for extrapolation of interindividual variability in DMET abundance to predict the effect of population variability on drug pharmacokinetics from healthy adult population data. The model structure is based on a middle-out approach, and the reported clinical PK study data or in-house experimental data can be used as input parameters (i.e., system- and drug-specific parameters). CL, intrinsic clearance; HA, healthy adults; SP, special populations such as pediatric, pregnant women, geriatric, and patients with organ dysfunction/disease state.

the poor translation of animal data. For example, biliary excretion of drugs or metabolites varies significantly in animals versus humans due to species differences in hepatic blood flow and bile flow along with transporter abundance, and it is difficult to predict drug or metabolites hepatic excretion of drugs and metabolites (Fallon et al., 2016). For example, an eightfold higher in vitro bile canalicular clearance of 2,4-dinitrophenyl-S-glutathione was observed in rats than dogs in hepatocytes (Ishizuka et al., 1999). Therefore, the characterization of interspecies differences in drug metabolism and transport is useful in (1) better translation of preclinical to clinical data, (2) distinguishing toxicity mechanisms, and (3) avoiding clinically relevant DDIs. Quantitative proteomics has been used to determine species differences in DMET proteins for better interpretation and scaling of animal data (Table 5). For example, the abundant expression of AO in human and monkey explain higher metabolite formation and toxicity in these species as compared with dogs and rats (Diamond et al., 2010; Basit et al., 2021). In particular, the ability to quantify multiple proteins and the high precision of the quantitative proteomics approach allows for an accurate determination of interspecies differences in DMET protein abundance, which can be integrated into the PBPK model to extrapolate PK parameters in humans. However, it is important to consider that not only the abundance of DMET proteins is different between animals and humans, but the $K_m$ can also vary due to differences in the amino acid sequence at the active sites. Therefore, when scaling the intrinsic clearance data from animal to human, both $K_m$ and abundance differences between the species must be considered as illustrated in eqs. 3–5. Where, $K_{cat}$ is the turnover number, i.e. the number of times each enzyme site converts substrate to product per unit time and [S] is the substrate concentration.

$$CL_{\text{int, animal}} = \left(\frac{V_{\max}}{K_m}\right)_{animal} = \frac{Enzyme\ abundance_a * K_{\text{cat}}}{K_{m;a}} \quad (3)$$

$$CL_{\text{int, human}}\left(= \frac{V_{\max}}{K_m}\right)_{human} = \frac{Enzyme\ abundance_h * K_{\text{cat}}}{K_{m;h}} \quad (4)$$

*Assume $K_{cat}$ is similar in human and animal*

$$CL_{\text{int, human}} = \frac{CL_{\text{int, animal}} * Enzyme\ abundance_h * K_{m;a}}{Enzyme\ abundance_a * K_{m;h}} \quad (5)$$

### *F. Protein Quantification in Liquid Biopsies and Extracellular Vesicles*

Biofluids such as blood and urine serve as liquid biopsies to characterize interindividual differences and offer a multitude of safety and logistical advantages due to the ease of access and the involvement of less or noninvasive procedures. Biomarker quantification in biofluids is a fundamental approach in drug discovery and development for monitoring drug effectiveness, safety, and toxicity. Since most biomarkers are proteins, quantitative proteomics has emerged as one of the most powerful techniques for biomarker discovery and quantification. For example, CD14 and α-fetoprotein were identified as potential biomarkers of hepatocellular carcinoma in high-risk cohort serum samples using iTRAQ-based quantitative proteomics (Guo et al., 2017). Similarly, the utility of quantitative proteomics in the characterization of protein induced by vitamin K antagonist-II (PIVKA-II) biomarker for blood clotting factor deficiency, vitamin K–related disorders, and diagnosis and monitoring of hepatocellular carcinoma (Basit et al., 2020). Further, the multiplex quantification of protein biomarkers in biofluids can provide information on changes in both individual proteins as well as biologic pathways perturbed

by a drug or disease, hence allowing for systems-level information.

Protein quantification in extracellular vesicles (EVs) secreted in blood or urine from individual tissues is emerging as a promising approach in precision medicine. EVs are small extracellular, nonreplicating nanovesicles that are considered a rich source of biomarkers. EVs contain a collection of proteins, DNA, mRNA, and lipids derived from the cell of origin. EVs are broadly classified into three classes based on their sizes, such as exosomes (50–150 nm), microvesicles (100–1000 nm), and apoptotic bodies (50–5000 nm) (Yáñez-Mó et al., 2015), and they can be distinguished from other EVs from their surface-expressed set of tetraspanins (CD9, CD63, and CD81). The EV isolation technique typically involves (1) ultracentrifugation, (2) size-exclusion chromatography, (3) resin precipitation, and (4) membrane affinity chromatography (Fig. 7) (Rodrigues et al., 2021). The immunocapture or immunoprecipitation methods are particularly useful because of their ability to selectively enrich exosomes from a specific tissue using antibodies against a marker protein that is exclusively expressed in the tissue of interest.

Besides monitoring disease severity and drug response, EVs have been recently used to quantify DMET proteins. For example, the quantification of DMET proteins in EVs has been successfully used to quantify the effect of genetic polymorphism (Rodrigues and Rowland, 2019), DDI (Rodrigues and Rowland, 2019), and overall interindividual variability (Achour et al., 2021). The total plasma exosomes from rifampicin-dosed individuals have shown a strong correlation between CYP3A4 exosomal expression (mRNA and proteins) versus midazolam oral clearance (Rowland et al., 2019). Similarly, rifampicin-mediated induction of CYP3A4 in HepaRG cells was precited by analyzing exosomes. The utility of quantitative proteomics in liquid biopsy analysis has also been shown for the determination of the effect of pregnancy on DMET abundance (Rodrigues et al., 2021), which revealed that CYP3A4 is induced; however, CYP2D6 and OATP1B1 showed no apparent induction during pregnancy. Other studies investigated alcohol-induced oxidative stress (Cho et al., 2017) and drug-induced toxicity (Kumar et al., 2017a) using CYP2E1 in EVs or plasma exosomes as a marker. Similarly urinary exosomes were used to quantify makers of IgA nephropathy (Moon et al., 2011) and thin basement membrane nephropathy (Rahman et al., 2019). Thus, liquid biopsy is a promising technique, which, in conjunction with quantitative proteomics, has potential for broader applications in biomarker research. In particular, the use of liquid biopsies can be applied for patient stratification based on their individual ability to metabolize or excrete drugs for precise drug dosing. Because of the noninvasive nature of the liquid biopsy approach, the technique can also be potentially applied to characterize the effect of chronic diseases and the long-term use of medication on DMET proteins.

## IV. Recommendations for the Reproducible Use of Quantitative Proteomics

Although quantitative proteomics is a promising technique to predict interindividual variability, these data can be confounded by the quality of tissue samples. Banked tissues can often be affected by confounding variables associated with harvesting, processing, and storage. Similarly, the presence of scarred tissue, medication use, and zonal (spatial) variability in DMET abundance can confound the determination of interindividual variability using banked tissues. Harmonized protocol of tissue collection and storage should be employed along

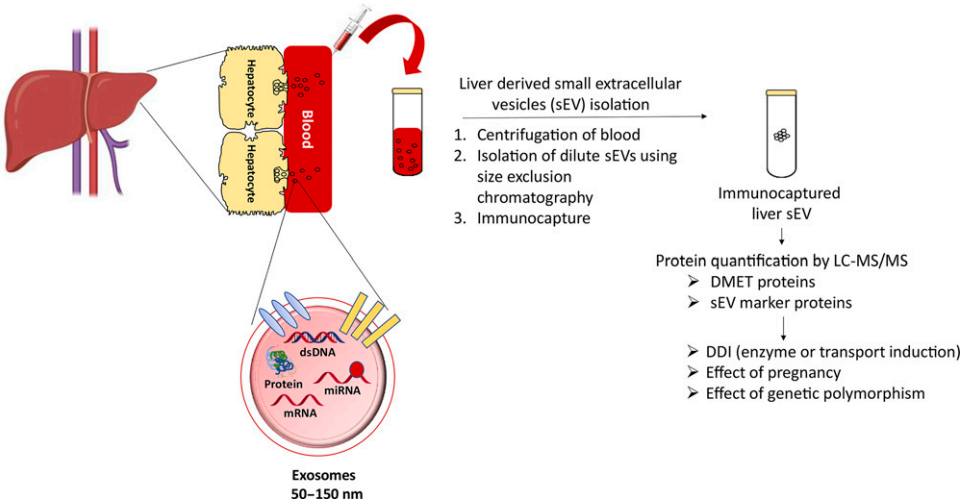


**Fig. 7.** Utilizing circulating small extracellular vesicles (sEVs) for predicting tissue DMET abundance for noninvasive prediction of drug disposition, DDI, and effect of genetic polymorphism. To isolate sEV as liquid biopsy from plasma, samples are centrifuge or enriched using size exclusion chromatography or immunocapture to obtain concentrated sEV collection from individual (Rodrigues et al., 2021).

with the collection of critical demographic and medical records to allow the comparison of data from different laboratories. In the case of a heterogenous tissue (e.g., placenta or kidney), the localization of DMET proteins is an important factor. For instance, the expression pattern of DMET proteins is different in the kidney cortex versus the medulla. Hence, to account for the contamination from the medulla in the cortex during fraction preparation, anatomic markers (e.g., aquaporins) can be used to account for the contamination.

The purity of subcellular fractions such as microsomes is often not characterized for contamination from other organelles, e.g., cytosolic contamination in microsomes. Quantitative proteomics can help in detecting contamination by measuring marker proteins, e.g., calnexin and calreticulin as markers of the ER membrane and ER lumen, respectively. CYPs are present in the ER membrane, and non-CYP enzymes are present in the cytosol or lumen of cellular organelles (Xu et al., 2018). Therefore, it is recommended that DMET proteins should be ideally quantified directly in tissue homogenate (Prasad et al., 2016). Optimization of tissue-specific homogenization methods, buffers, the volume of extraction buffer per gram of tissue, and optimized temperature conditions are important for generating reliable models. The use of EVs as a source of DMET protein quantification can be confounded by cell debris, which could be addressed by considering the shedding factor for normalization of DMET protein abundance (Achour et al., 2021).

Interday variability due to subtle changes in the sample preparation and LC-MS platforms may result in trypsin digestion variability. The use of an external protein standard such as bovine serum albumin as an internal control can resolve this issue (Bhatt et al., 2018). Solubility and stability of SIL peptides should also be checked and validated experimentally for the accurate estimation of peptide concentration in the biologic matrix. Also, contamination of light peptides in the heavy peptide standard should be checked to avoid false-positive results. Absolute protein abundance is affected by several factors such as trypsin digestion efficiency, quality of the calibrator peptides, and differential enrichment across laboratories. These challenges require that a common calibrator, for example, a well-characterized pooled human tissue sample, can be used to monitor such crosslaboratory variability. Digestion efficiency can also be assessed using codigestion of exogenously added proteins such as bovine serum albumin or QconCAT.

## V. Conclusions and Future Directions

Applications of quantitative proteomics in translational ADME and precision medicine have been increasingly reported in the recent past. With its ability to support IVIVE and PBPK modeling, as well as characterization of in vitro models, intertissue, and interspecies DMET protein variability and disease progression, quantitative proteomics serves as a key tool from early stages of drug discovery to clinical development and pharmacotherapy. Furthermore, an evolving trend of the use of quantitative proteomics in liquid biopsy (exosomes) allows the noninvasive quantification of DMET proteins to monitor real-time disease progression and therapeutic response. Increased sensitivity of LC-MS and miniaturized sample preparation are expected to be useful in the analysis of DMET proteins in the samples harvested using microsampling techniques. High interlaboratory variability in DMET proteomics data are a concern, which requires collective efforts to develop robust protocols and quality controls. Further, there is a need for harmonized protocols for biospecimen collection, archiving, sample preparation, and MS data analysis to avoid crosslaboratory variability. A universal control sample can be developed to be shared across sites to ensure interlaboratory reproducibility. Global proteomics data are emerging in the literature that can be archived in well-open access libraries for future use.

### Authorship Contributions

*Participated in research design:* Ahire, Prasad.

*Performed data analysis:* Ahire, Kruger, Sharma, Mettu, Basit, Prasad.

*Wrote or contributed to the writing of the manuscript:* Ahire, Kruger, Sharma, Mettu, Basit, Prasad.

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