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Characterization of Hepatic UDP-Glucuronosyl Transferase Enzyme Abundance – Activity Correlations and Population Variability using a Proteomics Approach and Comparison with Cytochrome P450 Enzymes

Ryan H. Takahashi, William F. Forrest, Alexander D. Smith, Justine Badee, NaHong Qiu, Stephan Schmidt, Abby C. Collier, Neil Parrott, Stephen Fowler

Department of Drug Metabolism and Pharmacokinetics (R.H.T.) and Department of OMNI Bioinformatics (W.F.F.), Genentech, Inc., South San Francisco, USA;

Department of Pharmaceutics, Center for Pharmacometrics and Systems Pharmacology (J.B., S.S.), University of Florida at Lake Nona, Orlando, Florida, USA.;

Pharmaceutical Research and Early Development, Roche Innovation Centre Basel, F. Hoffmann-La Roche Ltd. (N.Q., N.P., S.F.), Grenzacherstrasse 124, Basel, Switzerland;

Faculty of Pharmaceutical Sciences, The University of British Columbia (A.D.S., A.C.C.), Vancouver, BC, Canada.

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Running Title: UGT and CYP Proteomic Abundance-Activity Assessment

Corresponding author:

Stephen Fowler, Pharmaceutical Research and Early Development, Roche Innovation Centre Basel, F. Hoffmann-La Roche Ltd, Grenzacherstrasse 124, Basel, CH4070, Switzerland.

Phone: +41 61 688 5105

email: stephen.fowler@roche.com

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Abbreviations

CV, coefficient of variation; CYP, cytochrome P450; DTT, dithiothreitol; HLM, human liver microsomes; HPLC, high performance liquid chromatography; LC-MS/MS, liquid chromatography-tandem mass spectrometry; PK, pharmacokinetics; QC, quality control; rs, Spearman's rank correlation coefficient; SDC, sodium deoxycholate; SIL, stable isotope labeled; UGT, UDP-glucuronysyl transferase

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Abstract

The expression of ten major drug-metabolizing UDP-glucuronosyl transferase (UGT) enzymes in a panel of 130 human hepatic microsomal samples was measured using a LC-MS/MS-based approach. Simultaneously, ten cytochrome P450s and P450 reductase were also measured and activity-expression relationships assessed for comparison. The resulting data sets demonstrated that, with the exception of UGT2B17, 10th-90th percentiles of UGT expression spanned 3- to 8-fold ranges. These ranges were small relative to ranges of reported mean UGT enzyme expression across different laboratories. We tested correlation of UGT expression with enzymatic activities using selective probe substrates. A high degree of abundance-activity correlation (rs > 0.6) was observed for UGT1As (1A1, 3, 4, 6) and CYPs. In contrast, protein abundance and activity did not correlate strongly for UGT1A9 and UGT2B enzymes (2B4, 7, 10, 15 and 17). Protein abundance was strongly correlated for UGTs 2B7, 2B10 and 2B15. We suggest a number of factors may contribute to these differences including incomplete selectivity of probe substrates, correlated expression of these UGT2B isoforms, and the impact of splice and polymorphic variants on the peptides used in proteomics analysis and exemplify this in the case of UGT2B10. Extensive correlation analyses identified important criteria for validating the fidelity of proteomics and enzymatic activity approaches for assessing UGT variability, population differences and ontogenetic changes.

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Significance Statement

Protein expression data allow detailed assessment of inter-individual variability and enzyme ontogeny. We observed that expression and enzyme activity are well correlated for hepatic UGT1A enzymes and P450s. However, for the UGT2B family, caution is advised when assuming correlation of expression and activity as is often done in physiologically based pharmacokinetic modeling. This can be due to incomplete probe substrate specificities but may also be related to presence of inactive UGT protein materials and the effect of splicing variations.

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Introduction

The liver is often the most important organ for drug clearance given its high delivery of systemic blood, large organ mass and high expression of metabolizing enzymes (Williams et al., 2004; Cerny, 2016). The enzymes of highest interest, due to their predominant role in metabolizing small molecule drugs, belong to the cytochrome P450 (CYP) and UDP-glucuronosyl transferase (UGT) families (Cerny, 2016). There has been an apparent movement in the chemistries of candidate drug molecules away from primarily P450-mediated clearance and towards other enzymes (Argikar et al., 2016; Cerny, 2016). Thus, it is likely that UGT-mediated conjugation will be more frequently encountered in candidate drug molecules, potentially reducing drug-drug interaction liabilities due to lower time-dependent inhibition and induction risks, but also requiring better understanding of these enzymes to enable in vitro metabolism characterization and prediction of in vivo outcomes.

Heterogeneity in hepatic enzyme expression between individuals can contribute to variability in drug pharmacokinetics (PK) and can cause risk of underexposure or overexposure to a new drug or its metabolites in some individuals. This may result in lower drug efficacy or higher toxicity, especially for narrow therapeutic index drugs. Examples for UGT-cleared drugs that were recognized to have high interindividual variabilities are UGT2B10-metabolized RO5263397 (Fowler et al., 2015) and UGT2B17-metabolized MK-7246 (Wang et al., 2012). In vitro-in vivo extrapolation (IVIVE) embedded within physiologically-based pharmacokinetic (PBPK) models is widely employed to predict a drug's PK and variability within a given population (Rowland et al., 2011). IVIVE relies on the assumption that enzyme abundance is predictive of in vivo activity and population variability in enzyme abundance is a major contributor to projected in vivo clearance variability (Howgate et al., 2006). This IVIVE approach has been developed for

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CYP isoforms (Proctor et al., 2004) and PBPK models have been successfully verified for many CYP-cleared drugs. The IVIVE approach is less well established for UGTs (Jones et al., 2015) for which (compared with CYPs) fewer enzyme-selective probe substrates have been studied and over a shorter period of time. Recently, Docci et al. developed PBPK models for four UGT substrates, using available clinical data to overcome gaps in IVIVE (Docci et al., 2020). Their work also highlighted gaps and uncertainties in UGT abundance data which currently limit IVIVE and PBPK modelling for UGT substrates.

Quantification of enzymes in human tissue samples is typically performed by measuring enzymatic activities or protein concentration/expression. Measurement of both, using the same panel of samples, enables cross-comparison of the methodologies and enhances confidence when these parameters are strongly correlated. When UGT activities for a large number of individual donor liver microsomal samples were measured, population variability and enzyme activity development with age could be approximated (Badee et al., 2019; Liu et al., 2020). However, this approach consumes significant amounts of microsomal material and relies on the use of enzyme-selective probe substrates. Immunoblotting has historically been used for protein quantification, but this approach is resource-intensive, requiring each protein to be probed discretely. It can be limited by the availability of protein-specific antibodies and consumes large amounts of sample. As an alternate approach, LC-MS/MS monitoring and quantification of protein-specific surrogate peptides can be highly selective and sensitive, requires little material and is amenable to multiplexing for simultaneously measuring several proteins (Prasad et al., 2017). This has led to its adoption for quantifying key drug metabolizing enzymes and transport proteins and, in particular, has been applied to profiling large banks of individual donor samples (Bhatt and Prasad, 2018; Basit et al., 2020). With several laboratories having now reported LC-

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MS/MS quantifications of ADME proteins, it has become clear that differences exist in the methodologies employed and the expression levels reported (Achour et al., 2014a; Wegler et al., 2017; Achour et al., 2018). Whereas bioanalysis of new chemical entities or their metabolites follow stringent criteria for validation, there is still discussion on what constitutes best practice for proteomics approaches (Prasad et al., 2019).

In the current study, we quantified ten UGTs, encompassing the major drug metabolizing isoforms, in a collection of human liver microsome samples using a LC-MS/MS-based proteomics approach. The methods here built upon previously reported approaches and focused on enhancing tryptic peptide recoveries, normalizing for inter-batch variabilities, monitoring multiple peptides, and verifying the linearity via mixed human liver microsome (HLM) samples. The expression determinations were corroborated by comparing with enzymatic activities measured using probe substrates. The major hepatic drug-metabolizing CYPs were simultaneously monitored in these studies, which enabled us to compare and contrast observations for the less studied UGTs with those for well-characterized CYPs. The resultant data sets provide a rich description of the inter-individual differences in enzyme expression, activity and the correlation of these properties.

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Materials and Methods

Materials. Single donor human liver microsomes were obtained from commercial sources. 108 single donor lots were purchased from Sekisui Xenotech (Lenexa, KS) and twelve lots were purchased from Corning (Woburn, MA). HLM from ten single pediatric donors were provided by Abby C. Collier (University of British Columbia, Canada, Human Subjects Approval H14-0009). The 200-donor mixed gender pooled HLM used as a batch QC sample was purchased from Corning. All microsomes were kept stored at -80°C. Stable isotope labeled (SIL) peptide standards with ^{13}C and ^{15}N C-terminal lysine or arginine were purchased from New England Peptide (Gardner, MA) and CPC Scientific (Sunnyvale, CA). Peptide standards were received from the vendor with certificates of analysis confirming >95% peptide purity and >99% isotopic purity by amino acid analysis, MALDI, and HPLC analysis. SIL peptides were stored at -20°C dissolved in water:acetonitrile (70:30 v:v) containing 0.1% formic acid. Ammonium bicarbonate, CelLytic M, dithiothreitol, iodoacetamide, trypsin (sequencing grade) were purchased from Sigma (St Louis, MO). All other reagents were of analytical quality or higher.

Sample Demographics. The donor samples in this study were not selected to reflect a specific population but rather to represent a large range of livers that were available from commercial providers and research institute HLM collections. There were 83 male donors and 47 female donors. Most were of Caucasian origin (95) with a small number of African-American (20), Hispanic (9), and Asian (3) origin. Ethnicities for 3 donors were not available. Following on from research into the ontogeny of drug-metabolizing enzymes (Badée et al., 2019a; Badee et al., 2019), the sample set was somewhat enriched with pediatric donors. The donors ranged in age from 0.04 to 79 years of age with the following number of samples in each age group: 0-2 years, 18; 2-6 y, 8; 6-12 y, 9; 12-18 y, 7; 18-60 y, 70; and 60-80 y, 18.

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Trypsin Digestion of HLM Samples. A standard in-solution trypsin digestion protocol was optimized and then employed for this study. To allow batch analysis, sample preparation was completed using a 96-well plate format. The base protocol mixed microsomal samples (40 µg total protein, nominal protein concentrations provided by vendors were used) and dithiothreitol (10 mM) in ammonium bicarbonate buffer (22.5 mM) in a sample volume of 80 µl. Proteins were denatured by heating to 60°C for 1 h then allowed to cool to room temperature. Iodoacetamide was added as a solution in ammonium bicarbonate buffer (final 15 mM) and allowed to alkylate cysteine residues over 30 min in the dark at room temperature. Trypsin, dissolved in 50 mM acetic acid, was added to bring final sample volumes to 100 µl and the samples were incubated overnight covered at 37°C with shaking. At the end of the incubation, acetonitrile containing 2 pmol of $^{13}C^{15}N$-isotopically labelled synthetic peptide (internal standard) corresponding to each target protein was added to each sample. Samples were vortex mixed and centrifuged at 2,000 g at 4°C for 10 min. Supernatants were removed and concentrated under vacuum and the residues were redissolved to ~0.3x volume with water:acetonitrile (70:30) containing 0.1% formic acid.

To evaluate the effect of surfactant on trypsin digestion efficiency and peptide recoveries, detergents were added to the initial mixture of microsomes and dithiothreitol in buffer. The tested detergents were sodium deoxycholate (SDC), which was added dissolved in ammonium bicarbonate buffer to 10% (weight per volume); or CelLytic M, a commercial solution of proprietary detergents, which was added to 31.2% (by volume) or 62.5% (by volume). The trypsin amount providing optimal peptide generation was judged by completing digestions at 1:100, 1:50, and 1:20 trypsin:protein ratios in a single experiment. For each optimization

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condition (detergent or trypsin), triplicate digestions were completed with a single microsomal sample to evaluate the reproducibility of the digestion.

Batch analysis of the single donor HLM was completed using the base protocol with the following best tested conditions for digestion efficiency: addition of CelLytic at 62.5% (by volume) for denaturation and trypsin at 1:50 (ratio to protein). In each batch of samples, 4-6 replicates of 200-donor pooled HLM were included to act as QC samples. The 130 single donor HLM lots were prepared and analyzed in two analytical batches with each lot in singlicate and the QC sample in replicate.

LC-MS/MS Analysis. Trypsin digests were chromatographed on a Nexera UPLC system (Shimadzu, Kyoto, Japan) and analyzed by scheduled selected reaction monitoring (SRM) analysis with a QTRAP 5500 hybrid mass spectrometer operated using Analyst 1.5 software (Sciex, Framingham, MA). 10 µl of prepared sample, which contained tryptic digest of ~4 µg of total microsomal protein was injected to the column. Separations were completed on an Omega Polar PS-C18, 100×2.1 mm, 1.6 µm particle size, analytical column (Phenomenex, Torrance, CA) with a constant flow rate of 0.65 ml/min of mobile phases (A) 0.1% formic acid in water and (B) 0.1% formic acid in acetonitrile with a gradient elution program. The program started at 5% B, which was held for 2 min, then the %B was increased to 15% at 26 min, 20% at 38 min, and 45% at 46 min. The column was then washed at 98% B for 4 min before re-equilibration for 4 min at 5% B. The total run time was 55 min.

Method creation, data acquisition and processing were completed using Skyline software (MacLean et al., 2010). One peptide was used for each target protein for quantification with

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SRM transitions calculated by Skyline and confirmed in method development experiments. Doubly and triply charged precursor ions and three singly charged product ions were used to monitor each peptide (Supplemental Table 1). Optimal declustering potential (DP) and collision energies (CE) for each SRM transition were predicted by Skyline algorithms. In addition to SRMs for the quantifier peptide, at least one additional unique peptide from a distinct region of the protein was monitored as a qualifier peptide. In order to monitor all SRM transitions in a single sample injection and chromatographic run, each transition was scheduled for 3 min centered at the expected retention time for each peptide with a target scan time of 1 s.

Data were imported to Skyline for inspection and integration. Peak area ratios for the quantifier peptide were normalized to those of the corresponding SIL peptide and scaled to the amount of SIL added to calculate the protein amount in each sample. SIL standards were not obtained for qualifier peptides. The nominal concentrations provided by the vendor or calculated based on the reported amount provided for SIL peptides were used for calibration. The expression was expressed as pmol of protein determined from analysis per total microsomal protein that was added to the digestion. The microsomal protein amounts were based on vendor reported concentrations and were not re-measured. In each batch, a 200-donor pooled HLM lot was digested in replicate and analyzed alongside the single donor HLM. To account for batch effects, the observed response for each single donor HLM were normalized to the mean response for the 200-donor pooled HLM for each enzyme in the same batch.

Linearity of Methods. HLM linearity standards were created by mixing single donor HLM lots that had been characterized as having low and high expression. Six pairs of HLM lots were

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selected to span the ranges of protein expression for nine major drug-metabolizing enzymes (CYP1A2, 2B6, 2C9, 2D6, 3A4, UGT1A1, 1A4, 2B7, 2B15). For each pair, HLM were mixed at 1:0, 1:4, 2:3, 3:2, 4:1, and 0:1 volume ratios (low expression:high expression) maintaining a constant total protein concentration of 20 mg/ml, then a 40 µg aliquot was taken and processed in the same manner as used for all single donor HLM lots. Linearity plots were constructed by plotting SRM responses, normalized to SIL peptide, against the nominal concentrations (pmol mg^{-1}) using Prism v 8.0.1. (GraphPad Software, Inc., San Diego, CA) and linearity was judged based on the coefficient of determination (r^2) for the fitted regression line.

Comparisons to Previous Reports of UGT Expression. Values from ten reports relating to LC-MS/MS-based quantification of UGTs in human liver microsomes have been compared in this study (Ohtsuki et al., 2011; Harbourt et al., 2012; Fallon et al., 2013; Achour et al., 2014b; Groer et al., 2014; Sato et al., 2014; Margaillan et al., 2015; Yan et al., 2015; Nakamura et al., 2016; Bhatt et al., 2018). Most reports described analysis of panels of single donor samples (n=4 to 48). For comparison with the current study and for calculating interlaboratory range and variability, all samples were assumed to be drawn from the same human population and the mean reported value for individual UGT isoforms represented each study.

Correlation with Enzyme Activities. CYP activities data were provided in microsomal lot characterization data sheets and used with permission from Sekisui-Xenotech. UGT activities data were measured by monitoring the glucuronide metabolite formation of identified substrates using automated methods that have been previously described (Badée et al., 2019b). The UGT

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isoform selective substrates were β-estradiol, chenodeoxycholic acid, trifluoperazine, 5-hydroxytryptophol, propofol, zidovudine, amitriptyline, oxazepam and testosterone to characterize the activities of UGT1A1, 1A3, 1A4, 1A6, 1A9, 2B7, 2B10, 2B15 and 2B17, respectively (Badée et al., 2019b). In addition, glucuronide formation of UGT-metabolized drugs were monitored, namely raltegravir (UGT1A1), dolutegravir (UGT1A1), lamotrigine (UGT1A4), mycophenolic acid (UGT1A9), gemfibrozil (UGT2B4, UGT2B7); ketoprofen (UGT2B7; 1A3), and RO5263397 (UGT2B10). Where the same lot of single donor HLM had measurements for expression and activities for an enzyme, association of the two parameters was tested by Spearman correlation.

Statistical Analyses. Expression measurements of CYPs and UGTs were completed in two analytical batches. Each batch contained replicate measurements (n=4 or 6), performed as discrete sample preparations and analyses, of pooled human liver microsomes (XT-200) as a QC sample and to enable inter-batch normalization. Concentrations measured in batch 1 were not modified. Concentrations measured in batch 2 were adapted by multiplying each value for the individual HLM samples by a normalization factor, as follows:

$$Normalization\ factor = \frac{Mean\ measured\ enzyme\ concentration\ (Pooled\ HLM, Batch\ 1)}{Mean\ measured\ enzyme\ concentration\ (Pooled\ HLM, Batch\ 2)}$$

Measurement of UGT activities were completed in a separate laboratory than expression measurements as has been previously reported (Badee et al., 2019). Matched single donor HLM

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samples were used for expression vs. activity comparisons. Expression and activities of the single donor HLM did not follow Gaussian distribution so nonparametric statistical assessment was used. Correlation analysis was performed using Spearman rank order correlation test with p-values of t-statistics to assess correlations of data. Positive correlations with rs > 0.4 were described as strong when rs ≥ 0.6 and moderate when 0.6 > rs > 0.4. Linear regression analysis was carried out to assess the linearity of relationships and scatter of the data. Statistical analysis and graph generation was completed using R (version 4.0.0, R Core Team, 2020) and Prism version 8.1.2. (GraphPad Software, San Diego, CA).

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Results

Method Optimization. In developing methods, key areas that were evaluated were peptide selection, trypsin digestion efficiency, and robustness of the mass spectrometric analysis.

Several expected peptides were chosen per target protein at the outset of method testing and monitored through method development and optimization experiments. Peptides that had been previously reported for quantitative analysis of UGTs or CYPs were prioritized. Peptides were not used if they were not detected, showed poor peak shape, or showed reduced response when samples were reanalyzed 24 to 48 h after initial analysis. The stable isotope labeled (SIL) peptide for the most promising peptide per protein was obtained by commercial custom synthesis and monitored as the primary quantifier peptide. Other peptides that remained after triage were maintained in the LC-MS/MS method as qualifier peptides.

Pooled human liver microsomes were digested in four digestion buffers and three trypsin-to-protein ratios in a single experiment. Each buffer and trypsin condition was performed with three replicates. The highest observed peptide:SIL ratios were recognized as the maximal recoveries of peptides to select the preferred sample digestion conditions (Supplemental Figure 1). There was little difference observed when trypsin:protein ratio was increased from 1:100 to 1:50, but further increase to 1:20 introduced more variability in replicate samples. The use of surfactant is important to ensure solubilization of membrane-bound proteins or to provide access to proteins sequestered by a membrane layer; however, the addition of surfactant can inhibit the activity of trypsin. For P450s, there was little change observed when SDC or CelLytic detergent was added to the tryptic digestion. In contrast, the addition of SDC lead to ~4-fold increase of UGT peptides and CelLytic (added to 31.3% by volume) provided 5-9-fold increases in UGT peptides

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recovered. Peptide recoveries were higher with CelLytic than with inclusion of SDC, and therefore, it was used for all subsequent batch analyses. The choice of a proprietary detergent mixture, CelLytic, was based on convenience and the potential to apply the proteomics method to hepatocyte samples in future.

Scheduled MRM successfully allowed all of the peptides to be monitored in a single analysis of each sample. The target cycle time was set as 1 s. The chromatographic run was 55 min per sample. When run times were shortened significantly from this, there were not enough scans to properly quantify some peaks.

Comparison of Quantifier and Qualifier Peptides. Each UGT and P450 was quantified using a surrogate peptide for which the SIL standard was obtained. During analysis, other protein-selective peptides were monitored as qualifier peptides. The MS response (peak area abundances) for the quantifier and qualifier peptides were compared during the method development with a set of seventeen samples that ranged in UGT and P450 abundances. If the peptides were equally recovered from the protein and analytically robust, peaks areas were expected to correlate well. Good agreement between quantifier and qualifier peptides was seen for most proteins monitored in this study as judged by Spearman rank correlation (rs) of 0.80-0.99 (Table 1). Two exceptions with observed discordance for the monitored peptides were UGT1A3 and UGT2B10, which had low/no correlation (rs = 0.09 and 0.32, respectively). In the case of UGT1A3, the quantifier peptide (YLSIPTVFFLR) showed a dynamic range across the HLM samples indicating it likely captured inter-subject variability. In contrast, the qualifier peptide did not, potentially due to some isobaric background signal and was therefore not used.

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For UGT2B10, the quantifier (ANVIATALAK) and qualifier peptides (DTFWLPFSQEQEILWAINDIIR and GHEVTVLASSASILFDPNDSSTLK) have been previously described by other laboratories (Achour et al., 2014b; Groer et al., 2014). The peptide GHE… was not considered ideal and was used as a qualifier without the SIL standard because it was long (24 amino acids), contained asparagine which had potential to undergo deamidation, and contained the D67Y polymorphism site. In contrast ANV…K is also present in human UGT2B11 and UGT2B28, although low interference from these proteins with UGT2B10 is expected in human liver microsomes, as discussed later. There was a strong correlation of ANV…K with GHE…K (rs = 0.96) but weak correlation with DTF…R (rs = 0.32), and weak correlation of GHE…K with DTF…R (rs = 0.37). A limitation in our UGT2B10 studies is that the final peptide selection was completed later in method development and the full sample set was not available for measurements (n = 49 for expression analysis and n = 32 for enzymatic activity).

Native protein expression linearity assessment using HLM. Six pairs of single donor HLM were selected to represent the lowest and highest expressers of nine major drug metabolizing enzymes, namely UGTs 1A1, 1A4, 2B7 and 2B15 as well as CYPs 1A2, 2B6, 2C9, 2D6 and 3A4. These HLM pairs were mixed at six defined ratios to create mixed HLM samples that spanned the range of hepatic expression for these enzymes. Each mixed HLM sample was processed and analyzed with the same method that was applied to single donor HLM. Linearity curves spanned large ranges of protein expression, ranging from 13.3-fold for UGT2B7 to 815-fold for CYP1A2, and linearity (r^2) ranged from 0.808-0.955 (Supplemental Figure 1,

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Supplemental Table 2). There were no obvious dependences on enzyme or concentrations for scatter of residuals.

UGT and CYP Expression in HLM. The expression of UGTs and P450s in single donor HLM (n=130) and 200-donor pooled HLM are shown in Figure 1. Large inter-individual variability in expression was observed for most enzymes. The 10th and 90th percentiles were used to describe the characteristics of the population and avoid focusing on individual outliers (UGTs: Table 2, CYPs: Supplemental Table 3). UGTs other than UGT2B17 showed relatively small ranges in 10th-90th percentile expression (3- to 8-fold). The largest 10th-90th percentile range of all the studied enzymes was for UGT2B17 (79-fold range). However, this range is more reflective of the apparent bimodal distribution and cannot be compared directly with the largely monomodal distributions seen for other UGTs (Figure 1). The UGT2B17 observations were consistent with those previously described by Bhatt et al. with low expression in samples from donors 12 years and younger, and a greater incidence of high expression in males than in females from donors older than 12 years (Bhatt et al., 2019). Other enzymes with non-Gaussian distributions in expression that tended towards bimodal were CYP2C19, CYP2D6 and CYP3A5, which have previously identified to be polymorphic affecting expressed protein levels. Generally, the median expression for the single donors was similar to the 200-donor pooled lot (Table 2). Except for UGT2B17 the single donor mean and the pooled donor abundances differed by less than 50%. The difference for UGT2B17 was 2.1-fold.

The mean expression determined for each UGT in the panel of liver samples was compared to values found in literature (Figure 2). Ten UGT datasets (Ohtsuki et al., 2011; Harbourt et al., 2012; Fallon et al., 2013; Achour et al., 2014b; Groer et al., 2014; Sato et al., 2014; Margaillan et al., 2015; Yan et al., 2015; Nakamura et al., 2016; Bhatt et al., 2018) were used as comparators.

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The reported mean UGT expression levels ranged 10- to 100-fold, indicating large interlaboratory variabilities. Mean expression levels in this study were within the ranges of previously reported means for all of the enzymes, except UGT2B15 and UGT2B17. The UGT "pie" showing mean data from the current analysis showed the majority of hepatic UGT content being attributed to UGT1A1, 1A4, and 2B7 (Supplemental Figure 2).

Correlation of UGT Expression Profiles. Correlation was tested in abundance vs. abundance with pair-wise comparisons of individual UGT isoforms. Visual representation of the correlation matrix is shown as Figure 3. Strong protein expression correlations (Spearman correlation coefficient rs ≥ 0.6) were observed between UGT2B7-2B10 and UGT2B7-2B15. Correlations with 0.5 < rs < 0.6 were observed between the following pairs of UGTs: 1A4-2B4, 1A6-1A9, 1A6-2B10, 2B4-2B15, 2B10-2B15.

Association of Expression and Activities. Human liver microsomes from pediatric (n = 43) and adult (n = 43) donors were incubated under optimized incubation conditions to assess the activity rates of hepatic UGTs using a panel of 16 in vitro UGT probe substrates and clinically used drugs with each compound tested discretely (Badee et al., 2019; Badée et al., 2019b). 108 HLM lots were characterized for P450 activities by the supplier (Xenotech) using accepted chemical probes and monitoring the formation of isoform-selective metabolites. The Spearman correlation coefficients for the expected substrate reactions and matched enzyme expression are described in Table 3.

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P450 probe substrate activities correlated strongly with the measured expressions (rs = 0.61-0.91, Table 3). Associations for CYP1A2, CYP2B6, CYP2C8 and CYP3A4/5 were determined using two substrates per enzyme and showed superior correlations using phenacetin (compared with ethoxyresorufin), buproprion (compared with *S*-mephenytoin), and amodiaquine (in comparison to paclitaxel), respectively, reflecting greater enzyme selectivity for these probe reactions. CYP3A4 expression correlated more strongly with testosterone 6β-hydroxylation (rs = 0.94) than with midazolam 1'-hydroxylation (rs = 0.84), presumably due to the involvement of CYP3A5 in metabolizing midazolam (Williams et al., 2002; Tseng et al., 2014). Indeed, correlation of CYP3A5 was higher with midazolam metabolism (rs = 0.55) than testosterone metabolism (rs = 0.43), though both correlations were substantially lower than those for CYP3A4 with these activity probes, reflecting the lesser contribution of CYP3A5 to total metabolism.

UGT1A enzymes showed moderate-to-strong correlations of expression and activities (rs = 0.51-0.86, Table 3). Additional drugs known to have high enzyme selectivity were tested for UGT1A1 (dolutegravir and raltegravir), 1A4 (lamotrigine), and 1A9 (mycophenolic acid). The tested drugs showed comparable correlations with expression as the probe substrates, supporting that they are predominantly conjugated by the respective UGT1A enzymes, with minor contributions by other UGTs (Picard et al., 2005; Rowland et al., 2006; Kassahun et al., 2007; Argikar and Remmel, 2009; Reese et al., 2013; Liu et al., 2019). Correlation of UGT1A9 abundance with mycophenolic acid glucuronidation was reasonable (rs = 0.63), but with propofol, an accepted probe substrate for UGT1A9, the degree of correlation was lower than expected (rs = 0.52).

Weak-to-moderate correlations were observed for UGT2B activities and measured expressions (Table 3). UGT2B4 expression showed some correlation with gemfibrozil glucuronidation, a non-selective substrate (rs = 0.43), and was not improved when a UGT2B7 inhibitor was added

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(rs = 0.38). Multiple substrates were tested for UGT2B7 and they showed no correlation except zidovudine, which showed a moderate correlation (rs = 0.34). Amitryptyline activities showed moderate correlation with UGT2B10 expression (rs = 0.31) whereas the more selective substrate RO5263397 (Milani et al., 2020) showed higher correlation (r=0.53), and *S*-oxazepam activities showed low correlation with UGT2B15 expression (rs = 0.28). UGT2B17 abundance showed higher correlation with testosterone glucuronidation (rs = 0.58), potentially benefitting from a wider dynamic range of enzyme expression.

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Discussion

Ten major UGT enzymes (as our primary interest) and ten major drug metabolizing CYPs (as comparators) were monitored to test and validate our methodological approaches. CYPs were efficiently assessed for peptide release with simple denaturation, alkylation, and tryptic digestion. In contrast, adding detergent (CelLytic M) and increasing trypsin:protein ratios increased recoveries of UGT peptides. This was unsurprising as UGTs localize to the luminal face of microsomes and are likely to be poorly accessed by trypsin. The linearity of the method was confirmed by selectively combining single donor HLM samples to span the full range of expressions (Supplemental Figure 1). This strategy for linearity assessment adds to traditional approaches of standard additions of synthetic peptides (non-SIL and SIL) to surrogate blank or matched matrices. This approach is resource-sparing, simultaneously assessed linearity of trypsin digestion and LC-MS measurement, and was deemed appropriate for quantifying inter-individual variability for each enzyme.

We monitored multiple peptides for each target protein to indicate the consistency of our proteomics approach. Where two peptides are equally subject to tryptic digestion efficiency and recovery, polymorphic variation, post-translational modification, and stability in analysis, the ratio of peptides arising from the same protein should be fixed and their abundances should correlate. This occurred for almost all peptide pairs (Table 1), with notable exceptions being UGT1A3 and UGT2B10. For UGT1A3, the peptide EVSVVDILSHASVWLFR showed a small dynamic range, indicating this peptide was not suitable. Fallon *et al* reported lack of sensitivity using this peptide (Fallon et al., 2013). A more successful approach has been monitoring the peptide YLSIPTVFFLR (Achour et al., 2017; Achour et al., 2018; Khatri et al., 2019). Our analysis using this peptide resulted in highly correlated enzyme concentration with activities

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measured using chenodeoxycholic acid (rs = 0.85). For UGT2B10; the peptide ANVIATALAK was selected and analyzed using its SIL analog after initial experiments using peptide DTFWLPFSQEQEILWAINDIIR showed its limitations. Some reports describe GHEVTVLASSASILFDPN**D**SSTLK as a preferred peptide for quantification (Fallon et al., 2013; Khatri et al., 2019) although it spans the D67Y polymorphism site. As shown in Figure 4, these peptides are regionally distinct, so their release with trypsin treatment should not be linked. Unfortunately ANV…K cannot be regarded as a completely selective peptide for UGT2B10 as this sequence is also present in human UGT2B11 and UGT2B28 which share 90 and 89% amino acid sequence identity, respectively, with UGT2B10 (Ohno and Nakajin, 2011). UGT2B28 has been reported to have little or no hepatic expression and is therefore unlikely to have interfered with UGT2B10 analysis (Nakamura et al., 2008; Ohno and Nakajin, 2011). Some contribution from UGT2B11 may have added an additional signal into the proteomics measurement, reducing the abundance-activity correlation. Anecdotally, the poor metabolizer phenotype donor H0295 (Milani et al., 2020) gave an extremely low but non-zero UGT2B10 abundance signal which may be indicative of background ANV…K signal coming from other sources in liver microsomes. GHE…K abundance correlated very highly with ANV…K (rs=0.956) further indicating a relatively low level of signal contamination in the liver microsomal samples assessed.

Assessment of large numbers of individual donor samples often requires batching of samples. We accounted for batch effects by normalizing to a QC sample that was included for each preparation and analysis. Our QC sample was a 200-donor pooled human liver microsomal sample (XT-200, Xenotech). A QC normalization approach becomes more valuable if a consensus sample is used across analysts, laboratories, and time, as demonstrated in a meta-analysis of UGT abundance studies using immunoblotting approaches (Liu et al., 2020).

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Here, we showed in a large liver panel that population mean expression values vary maximally 2-fold from that of the pooled liver microsomal samples and the 10th -90th percentile ranges were 8-fold or less (excepting UGT2B17). In contrast, 10-100-fold differences in mean concentrations were found between studies (Figure 3). Though inherent to interlaboratory variability is the analysis of different liver samples, the current data set indicates that the large range in reported values is methodological (different experimental and analytical protocols) in its origin rather than population variability.

CYP analyses were used to anchor method quality, validate the strategy of correlating enzyme expression with activity, and generate additional data on CYP expression distribution. Strong correlations (rs = 0.6 - 0.9) were observed, confirming the simple "more enzyme equals more activity" assumption and validating our methodological approach. For UGTs, a more complex picture emerged. Strong correlations were observed for UGT1A1, 1A3, 1A4, and 1A6 (rs > 0.75) but there was less association of measured expression with activities for the other studied UGTs. One limiting factor for correlation is the dynamic range of the measured UGT concentrations. The 10th-90th percentile ranges were relatively small, potentially limiting the obtainable correlation coefficients when taking into account inaccuracies in enzyme activity and enzyme concentration measurements.

We observed little correlation of enzymatic activites with expression for UGT2B7, UGT2B10 and UGT2B15 despite using validated substrates for these enzymes. The low correlations for UGT2B7 were surprising in light of previous proteomics-activity correlation reports of rs = 0.82 (Margaillan et al., 2015) or rs = 0.79 (Achour et al., 2017) and rs = 0.52 (Achour et al., 2018), where ziduovudine was used as probe substrate, as well as ketoprofen and zidovudine activities being highly correlated in our previous work (Badée et al., 2019b). Whilst additional UGTs other

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than UGT2B15 may contribute to S-oxazepam glucuronidation and account for lower expression-activity correlation (rs = 0.28), glucuronidation of RO5263397 at the concentrations tested is expected to be almost exclusively UGT2B10-mediated, and higher expression-activity correlation than observed (rs = 0.53) was expected. Numerous studies have explored the potential for multiple UGTs to contribute to glucuronidation of probe substrates, dependent upon substrate concentration and reaction conditions (Lv et al., 2019; Miners et al., 2020). We noted that the highest intercorrelation in expression of UGTs across the single donor liver samples existed for UGT2B7, 2B10, and 2B15 (Figure 3, rs ranging 0.59 to 0.82).

Proteomics approaches may not differentiate functional, incompletely expressed and non-functional proteins resulting from genetic variation. The detection or non-detection of some variants could disrupt activity-abundance correlations. This has highest potential to impact measurements of UGT2B isoforms. Unlike the UGT1A family, the UGT2B genes are produced from distinct gene loci with no shared exons. Exons 1 and 2 of each UGT2B gene code approximately the first 290 amino acids roughly corresponding to the substrate selective N-terminal end, with exons 3-5 mostly coding the more conserved sugar binding domain (Hu et al., 2019). The UGT2B genes encode between 7 and 44 alternatively spliced transcripts depending on the gene and varying based on tissue and disease state (Rouleau et al., 2016; Tourancheau et al., 2016). High levels of alternatively spliced transcripts for UGT2Bs (UGT2B4: 16%, UGT2B7: 8%, UGT2B10: 62%, UGT2B15: 6%, UGT2B17: 37%) have been found in the liver (Tourancheau et al., 2018).

UGT2B10 is a good example of this genetic variation and its potential impact in proteomics approaches (Figure 4). Including the canonical sequence, there are eleven UGT2B10 splice variants (Rouleau et al., 2016; Tourancheau et al., 2016; Tourancheau et al., 2018), all of which

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would be captured by at least one of the reporter peptides (Figure 4). There are also over 5000 SNPs present in the reference sequence, 638 of which result in protein alterations, including 78 located in the regions detected by the peptides. Peptide ANV…K has the fewest protein altering SNPs (14), whereas DTF...R (42 SNPs) and GHE…K (22 SNPs) have more protein-altering SNPs at their locations. As ANV…K is closest to the C-terminal end of the protein, it is less likely to be affected be frameshift or protein truncating mutations that result in non-functional proteins. Two UGT2B10 SNPs have been explicitly associated with a clinically relevant phenotype. The UGT2B10*2 allele (rs61750900, Asp67Tyr), present in ~11% of Caucasians (which constitute the majority of donor samples in this study) and ~5% of African Americans, is located within the region captured by the GHE…K peptide, and results in functional impairment (Chen et al., 2008; Berg et al., 2010; Bloom et al., 2013; Sipe et al., 2020). A second polymorphism (rs2942857) located at the splice-acceptor site between intron 2 and exon 3, present in ~0.4% of Caucasians and ~36% of African Americans, is believed to produce an unstable mRNA that fails to produce a viable protein. Individuals homozygous for this SNP present a UGT2B10 null phenotype and exhibit essentially no UGT2B10-related drug clearance (Fowler et al., 2015; Labriet et al., 2018; Sipe et al., 2020).

Additional alignments of UGT2B sequences and exon maps indicate that most polymorphisms would be captured by our reporter peptides. UGT2B7 has the largest number of splice variants of any UGT at 44, with only the canonical product being shown to have any functional activity to date, despite at least seven different proteins being produced from the first 22 splice variants alone (Ménard et al., 2009; Tourancheau et al., 2018). The peptides would capture 36 of the possible variants. The nine UGT2B15, and at least eight of the 10 UGT2B17 splice variants, would also be captured by at least one of the peptides for their respective products. Exceptions

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are polymorphisms not producing protein from any of the canonical exons, such as the UGT2B17*2 allele, common in Asian populations (Jakobsson et al., 2006). We recognize the potential for future proteomics studies to capture nonsynonymous or splice variant distribution, in addition to expression distribution, with strategically-selected peptides.

In this study, we applied LC-MS/MS methodologies to quantify hepatic UGTs. With the exception of UGT2B17, 10th-90th percentile ranges of UGT expression were between 3- and 8-fold and smaller than indicated by interlaboratory ranges. Combined proteomics and activity measurements provided new insights and revealed complexities of UGT enzymology and methodologies. These findings will be important for accurately translating UGT characterization data to estimates of drug clearance, population variability and enzyme ontogeny and should be bourne in mind by constructors of PBPK models for UGT substrates.

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Author Contributions

Participated in research design: Takahashi, Fowler

Conducted experiments: Takahashi

Contributed new reagents or analytic tools: Forrest

Performed data interpretation: Takahashi, Forrest, Badee, Fowler, Qiu, Collier, Parrott, Schmidt,

Contributed to the writing of the manuscript: Takahashi, Fowler, Smith, Collier, Parrott, Badee, Schmidt

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Footnotes

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Current Affiliations

Current affiliation for R.H.T. is Denali Therapeutics, South San Francisco, CA.

Current affiliation for J.B. is Department of PK Sciences, Novartis Institutes for BioMedical Research, Basel, Switzerland.

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Figure Legends

Figure 1. Frequency distribution of UGT and P450 expression within the set of liver samples under study. Blue solid line shows spline fit for the distribution, red dashed line shows median of results, and dotted red lines show 10% and 90% percentiles of results.

Figure 2. Mean UGT Abundance Reported in Various UGT Proteomics Studies. Comparison of hepatic expression levels measured in the current study (mean of single donor, n=130 (filled circles), and 200-pooled human microsomes (open circles)) with previous reports for UGT enzymes (Ohtsuki et al., 2011; Harbourt et al., 2012; Fallon et al., 2013; Achour et al., 2014b; Groer et al., 2014; Sato et al., 2014; Margaillan et al., 2015; Yan et al., 2015; Nakamura et al., 2016; Bhatt et al., 2018).

Figure 3. Correlation matrix of UGT enzymes (expression vs expression) observed with single donor human liver microsomes. Values are Spearman correlations (rs). Expression data for each enzyme describes 130 single donors except for UGT2B10, which was 49 single donors.

Figure 4. Diagram of the UGT2B10 amino acid sequence illustrating the locations of the proteomic reporting peptides, amino acid polymorphisms, and key splice variants. Red bars underneath the diagram of the canonical protein indicate regions that would be absent in the proteins encoded by the splice variants identified to date. The intronic SNP rs2942857 is predicted to produce an unstable mRNA that would be degraded before the protein is produced. Splice variants incorporating alternate versions of exon 6 are colored to match the exon map. The signal peptide (SP) is indicated in tan, the substrate binding domain in dotted green, the UDPGA

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co-substrate binding domain in cyan, the C-terminal region in purple, and the transmembrane domain (TM) in silver. †Variant n4 corresponds to NCBI GenBank isoform 2 (NP_001138239.1). ‡Variant n2 corresponds to NCBI GenBank isoform 3 (NP_001277020.1).

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Tables

Table 1. Spearman correlations for mass spectrometric responses (peak area abundances) of monitored quantifier and qualifier peptides for UGT enzymes.

Protein Target	Quantifier Peptide	Qualifier Peptide(s)	Correlation	
			Sample n	Spearman rs
UGT1A1	DGAFYTLK [1,2,3,4,5]	GHEIVVLAPDASLYIR [4]	16	0.959
		TYPVPFQR [2,4,6]	17	0.838
		EVTVQDLLSSASVWLFR [7]	17	0.865
UGT1A3	YLSIPTVFFLR [1,2,3,4,5,6]	EVSVVDILSHASVWLFR [4]	17	0.091
UGT1A4	YLSIPAVFFWR [1,3,4,5,6]	VTLGYTQGFFETEHLLK [4]	16	0.900
UGT1A6	DIVEVLSDR [1,3,4,5,6]	SFLTAPQTEYR [2,4]	15	0.986
		DVDIITLYQK [3]	16	0.979
UGT1A9	AFAHAQWK [3,4,6]	GHEVVVVMPEVSWQLGR	16	0.867
		ESSFDAVFLDPFDNCGLIVAK [2]	17	0.703
UGT2B4	FSPGYAIEK [1,2,3,4,5]	TILDELVQR	16	0.974
		ANVIASALAK [2]	17	0.956
UGT2B7	ADVWLIR [1,2,4,5]	TILDELIQR [2,3,6]	16	0.906
		IEIYPTSLTK [4]	13	0.962
		ANVIASALAQIPQK	15	0.886
UGT2B10	ANVIATALAK [8]	GHEVTVLASSASILFDPNDSSTLK [4,5]	17	0.956
		DTFWLPFSQEQEILWAINDIIR [2]	17	0.321
UGT2B15	FSVGYTFEK [1,4,6]	WIYGVSK [2]	16	0.900
		SVINDPVYK [2,3,4]	16	0.982
UGT2B17	FSVGYTVEK [4,5]	None	ND	ND

[1] Achour, 2017; [2] Achour, 2018; [3] Bhatt, 2018; [4] Fallon, 2013; [5] Khatri, 2019, [6] Sato, 2014, [7] Peptide also present in UGT1A7 sequence, [8] Peptide also present in UGT2B11 and 2B28 sequences.

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Table 2. UGT expression measured for single donor and 200-donor pooled human liver microsomes by LC-MS/MS. For single donor HLM, 10th, 50th (median), and 90th percentiles, and range are presented to describe the study's population data and mean is presented to compare to the pooled HLM. Coefficient of variation (CV, %) for pooled HLM is presented to describe measurement variability and represents n=4 replicates in one analytical batch.

Enzyme	Expression (pmol/mg)						
	Single Donor HLM (n=130 except for UGT2B10, which was n=49)				Single Donor HLM (n=130)	200-Donor Pooled HLM (n=4 measurements)	
	Percentile10	Median	Percentile90	Range (90:10 percentiles)	Mean	Mean	CV (%)
UGT1A1	35.2	80.7	165	4.7	102	68.5	8.63
UGT1A3	2.72	7.64	21.9	8.1	9.76	6.07	5.70
UGT1A4	29.1	67.4	114	3.9	69.2	67.9	7.02
UGT1A6	8.24	19.5	37.2	4.5	23.3	20.2	9.45
UGT1A9	9.22	15.0	26.8	2.9	16.2	15.8	9.71
UGT2B4	22.7	36.1	59.9	2.6	39.8	31.9	3.84
UGT2B7	26.9	61.2	111	4.1	66.7	45.5	10.0
UGT2B10	9.94	23.8	44.7	4.5	27.5	12.3	4.51
UGT2B15	7.41	12.6	29.7	4.0	16.2	11.0	22.1
UGT2B17	0.170	0.455	13.4	79	4.63	2.23	11.4

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Table 3. Correlation of enzyme expression with enzymatic activities measured with probe substrate reactions.

Enzyme	Reaction / Substrate	Spearman correlation (rs)	n
UGT1A1	Dolutegravir	0.795	62
	17β-Estradiol (3OH-glucuronidation)	0.782	62
	Raltegravir	0.783	79
UGT1A3	Chenodeoxycholic acid	0.846	62
UGT1A4	Trifluoperazine	0.729	62
	Lamotrigine	0.793	62
UGT1A6	5-Hydroxytryptofol	0.856	62
UGT1A9	Propofol	0.512	62
	Mycophenolic acid	0.587	79
UGT2B4	Gemfibrozil	0.433	62
	Gemfibrozil (with inclusion of UGT2B7 inhibitor)	0.379	62
UGT2B7	Zidovudine	0.344	62
	Ketoprofen	0.172	62
UGT2B10	RO5263397	0.529	32
	Amitriptyline	0.308	32
UGT2B15	(*S*)-Oxazepam	0.283	63
UGT2B17	Testosterone	0.582	62
CYP1A2	Phenacetin O-dealkylation	0.839	65
	Ethoxyresorufin O-deethylation	0.804	43
CYP2A6	Coumarin 7-hydroxylation	0.864	107
CYP2B6	(S)-Mephenytoin N-demethylation	0.696	84
	Bupropion hydroxylation	0.916	20
CYP2C8	Paclitaxel 6-alphahydroxylation	0.768	37
	Amodiaquine N-dealkylation	0.830	64
CYP2C9	Diclofenac 4'-hydroxylation	0.734	107
CYP2C19	(S)-Mephenytoin 4'-hydroxylation	0.876	99
CYP2D6	Dextromethorphan O-demethylation	0.853	107
CYP2E1	Chlorzoxazone 6-hydroxylation	0.758	107
CYP3A4	Testosterone 6β-hydroxylation	0.923	107
	Midazolam 1'-hydroxylation	0.837	63
CYP3A5	Testosterone 6β-hydroxylation	0.426	107
	Midazolam 1'-hydroxylation	0.550	63

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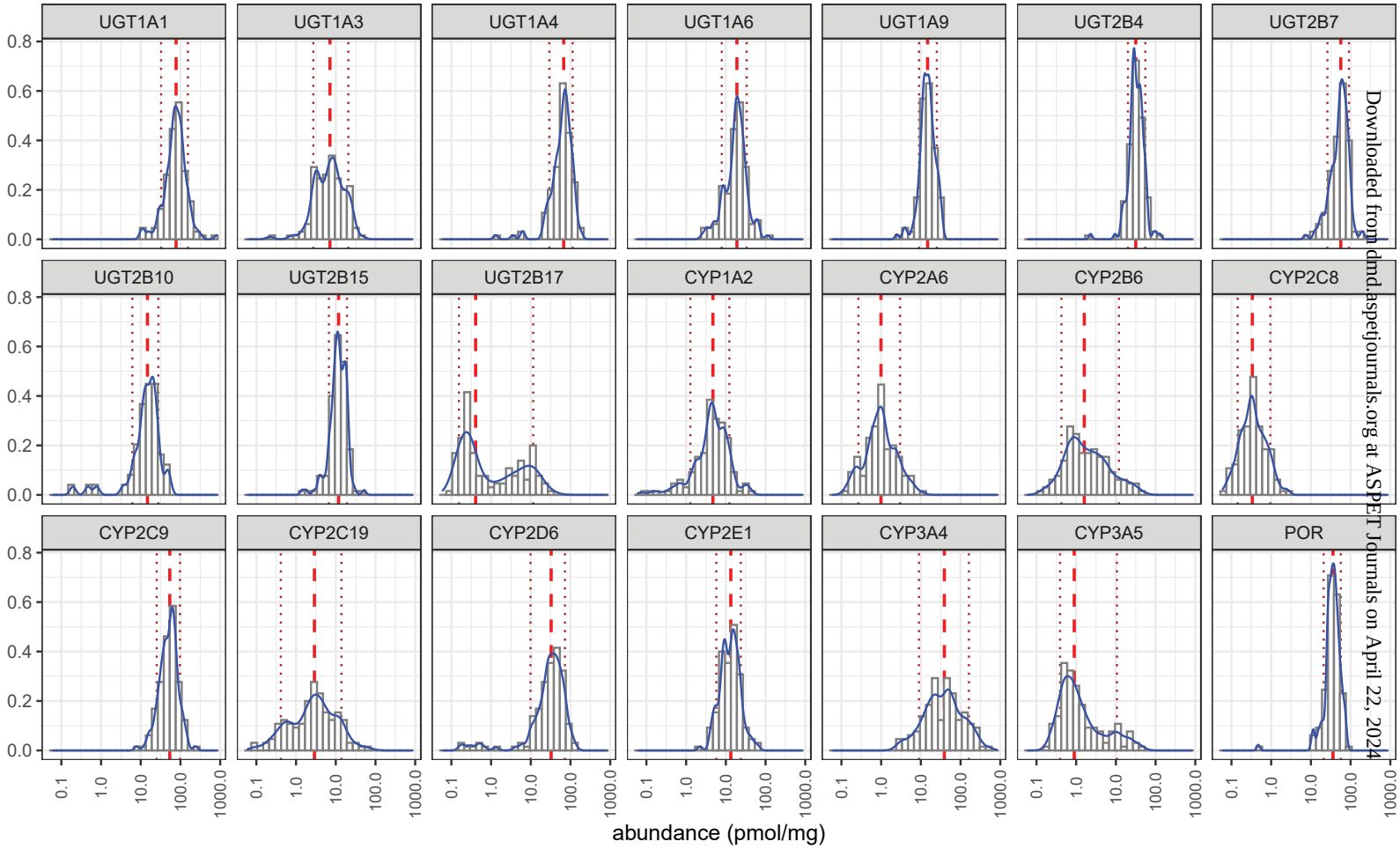

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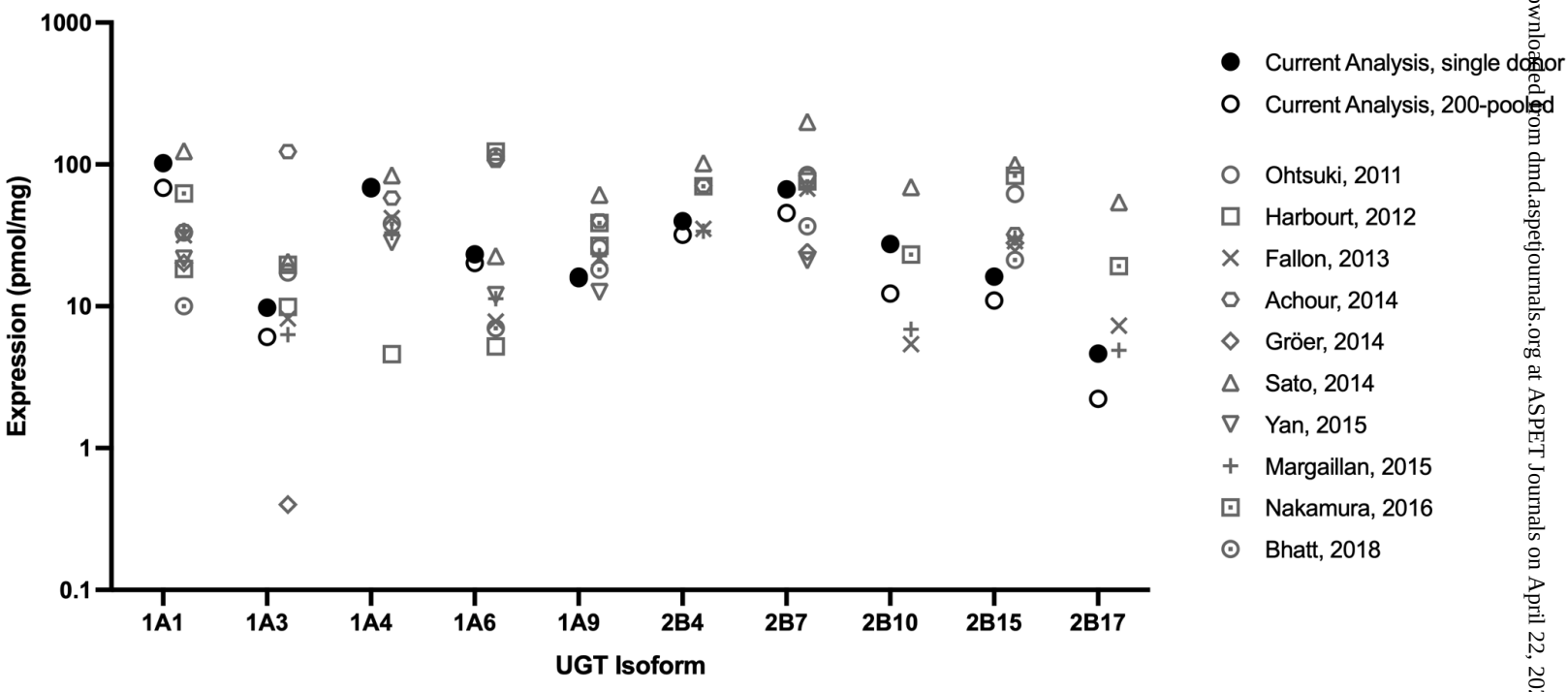

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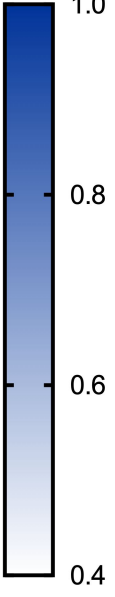
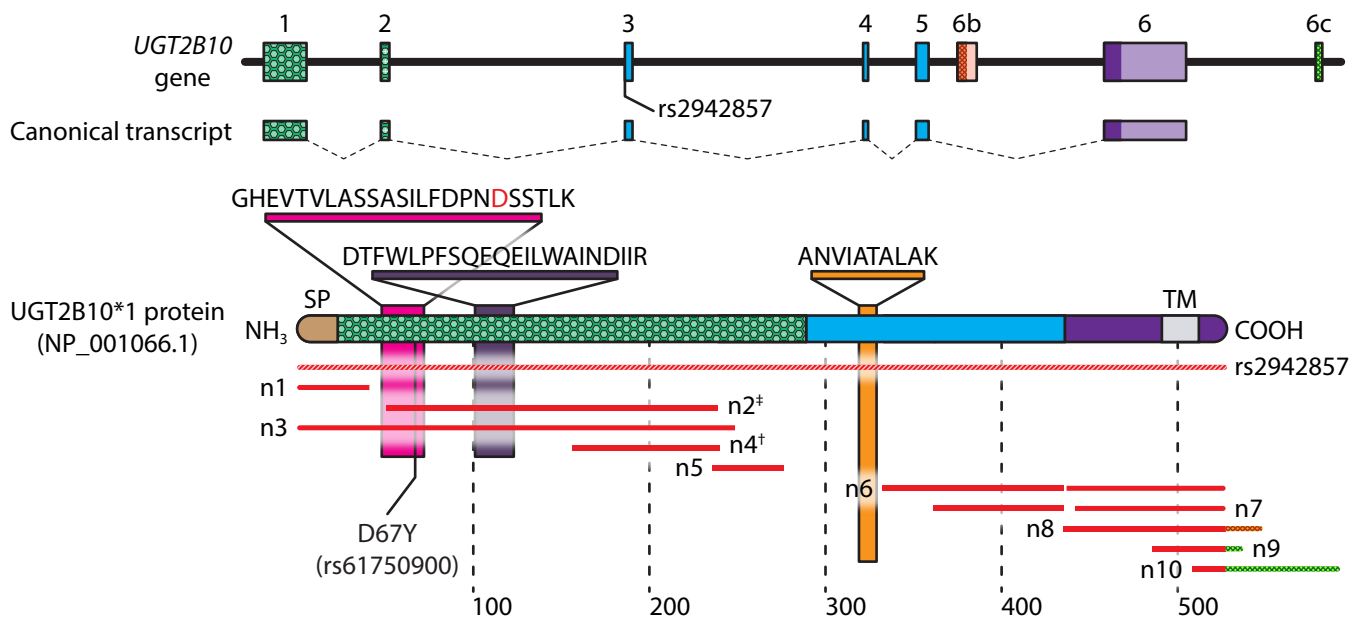
	UGT1A1	UGT1A3	UGT1A4	UGT1A6	UGT1A9	UGT2B4	UGT2B7	UGT2B10	UGT2B15	UGT2B17
UGT1A1	1.000	0.029	0.140	0.388	0.225	0.537	0.204	0.247	0.379	0.108
UGT1A3	0.029	1.000	0.324	0.020	0.140	0.171	-0.141	0.065	-0.155	-0.209
UGT1A4	0.140	0.324	1.000	0.290	0.426	0.442	0.300	0.354	0.188	0.123
UGT1A6	0.388	0.020	0.290	1.000	0.495	0.382	0.387	0.534	0.363	0.341
UGT1A9	0.225	0.140	0.426	0.495	1.000	0.323	0.301	0.452	0.229	0.104
UGT2B4	0.537	0.171	0.442	0.382	0.323	1.000	0.458	0.517	0.600	-0.004
UGT2B7	0.204	-0.141	0.300	0.387	0.301	0.458	1.000	0.762	0.835	0.384
UGT2B10	0.247	0.065	0.354	0.534	0.452	0.517	0.762	1.000	0.628	0.423
UGT2B15	0.379	-0.155	0.188	0.363	0.229	0.600	0.835	0.628	1.000	0.242
UGT2B17	0.108	-0.209	0.123	0.341	0.104	-0.004	0.384	0.423	0.242	1.000




UGT2B10 gene
1
2
3
4
5
6b
6
6c
rs2942857
Canonical transcript
GHEVTVLASSASILFDPNDSSTLK
DTFWLPFSQEQEILWAINDIIR
ANVIATALAK
UGT2B10*1 protein (NP_001066.1)
SP
TM
NH3
COOH
rs2942857
n1
n2‡
n3
n4†
n5
n6
n7
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n9
n10
D67Y
(rs61750900)
100
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