DMD Fast Forward. Published on November 7, 2019 as DOI: 10.1124/dmd.119.089235
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DMD # 89235

Comparative Proteomics Analysis of Human Liver Microsomes and S9 Fractions

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DMD # 89235

Running title: Comparative Proteomics of Human Liver Microsomes and S9

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Number of words in Abstract: 250

Number of words in Introduction: 776

Number of words in Discussion: 2304

Non-standard Abbreviations: DMEs, drug metabolism enzymes; HLM, human liver microsomes; HLS9, human liver S9 fractions; CYPs, cytochrome P450s; UGTs, uridine 5'diphospho-glucuronosyltransferases; APQ, absolute quantitative proteomics; DIA, data-independent acquisition; TPA, total protein approach; SILAC, stable isotope labeling using amino acid in cell culture; iTRAQ, isobaric tags for relative and absolute quantitation; DTT, dl-dithiothreitol; TFA, trifluoroacetic acid; IAA, iodoacetamide; Lys-C, lysyl endopeptidase; PBS, phosphate-buffered saline; BSA, bovine serum albumin; S9PPGL, s9 protein per gram liver; MPPGL, microsomal protein per gram liver; CV, coefficients of variation; CES1, carboxylesterase1; EPHX1, epoxide hydrolase1; EPHX2, epoxide hydrolase2; TPMT, thiopurine S-methyl transferase; COMT, catechol-O-methyltransferase; GST, enzymes from the glutathione S-transferase; SULT, sufotransferase; NAT, N-acetyltransferase; MGST, microsomal glutathione S-transferases; ABC, ATP-binding cassette; SLC, solute carrier family; ACTB, β-actin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; TUBB, β-tubulin; ER, endoplasmic reticulum; ATP1A1, ATPase subunit alpha-1; COX4I1, cytochrome c oxidase subunit 4 isoform 1; RPL7A, 60S ribosomal protein L7a; PSMD1, 26S proteasome non-ATPase regulatory subunit 1; FTCD, formimidoyltransferase cyclodeaminase; TMSB4X, thymosin beta-4; AKR1A1, aldo-keto reductase family 1 member A1; MRM, multiple-reaction monitoring; PONs, paraxonases

Abstract

Human liver microsomes (HLM) and S9 fractions (HLS9) are commonly used to study drug metabolism *in vitro*. However, a quantitative comparison of HLM and HLS9 proteomes is lacking, resulting in the arbitrary selection of one hepatic preparation over another and in difficulties with data interpretation. In this study, we applied a label-free global absolute quantitative proteomics method to the analysis of HLS9 and the corresponding HLM prepared from 102 individual human livers. A total of 3,137 proteins were absolutely quantified, and 3,087 of those were determined in both HLM and HLS9. Protein concentrations were highly correlated between the two hepatic preparations ($R=0.87$, $P<0.0001$). We reported the concentrations of 98 drug-metabolizing enzymes (DMEs) and 51 transporters, and demonstrated significant differences between their abundances in HLM and HLS9. We also revealed the protein-protein correlations among these DMEs and transporters and the sex effect on the HLM and HLS9 proteomes. Additionally, HLM and HLS9 displayed distinct expression patterns for protein markers of cytosol and various cellular organelles. Moreover, we evaluated the interindividual variability of three housekeeping proteins, and identified five proteins with low variation across individuals that have the potential to serve as new internal controls for Western blot experiments. In sum, these results will lead to a better understanding of data obtained from HLM and HLS9 and assist in *in vitro*-*in vivo* extrapolations. Knowing the differences between HLM and HLS9 also allows us to make a better-informed decision when choosing between these two hepatic preparations for an *in vitro* drug metabolism study.

Significance Statement

This investigation revealed significant differences in protein concentrations of numerous drug-metabolizing enzymes and transporters between human liver microsomes and S9 fractions. The study also determined the protein-protein correlations among the drug-metabolizing enzymes and transporters and the sex effect on the proteomes of these two hepatic preparations. The results will help interpret data obtained from these two hepatic preparations and also allow us to make a more informed decision when choosing between human liver microsomes and S9 fractions for an *in vitro* drug metabolism study.

Introduction

The liver plays a central role in the metabolism and disposition of various endogenous and foreign compounds (Meyer, 1996). Given the significant impact of hepatic metabolism and disposition on a drug's efficacy and toxicity, preclinical investigations are routinely performed using *in vitro* hepatic models (Iwatsubo et al., 1997). Several *in vitro* models have been developed to study hepatic drug biotransformation, which include recombinant drug-metabolizing enzymes (DMEs), liver subcellular fractions (microsomes, cytosol, and S9 fractions), hepatic cell lines, primary hepatocytes, liver slices, and perfused livers (Ekins et al., 2000; Brandon et al., 2003). Among these, human liver microsomes (HLM) and S9 fractions (HLS9) are the most commonly used *in vitro* models (Asha and Vidyavathi, 2010). Specifically, HLS9 are the supernatants of human liver homogenates after centrifugation at 9,000 ×*g* for 20 min, and consist of HLM and cytosol; HLM are the sediment separated from HLS9 by a further centrifugation at 100,000 ×*g* for ~1 hour. HLM are enriched with the membranes of the endoplasmic reticulum (ER) and other organelles, in which many DMEs and transporters are located (Fujiki et al., 1982; Ekins et al., 2000).

Hepatic DMEs and transporters are the essential element in determining drug metabolism and disposition. The protein expression levels of hepatic DMEs and transporters greatly impact pharmacokinetics, and are of importance for drug development. Thus, characterizing protein expression profiles in HLM and HLS9 would facilitate appropriate selection of these models in the study of drug metabolism as well as improve the interpretation of results from HLM and HLS9

incubation studies. A few attempts have been made to determine protein expressions in HLM, with a focus on DMEs and transporters (Ohtsuki et al., 2012b; Achour et al., 2014b; Golizeh et al., 2015a; Achour et al., 2017a; Achour et al., 2017b; Couto et al., 2019); however, HLS9 protein profiles have been largely inconclusive. In addition, most prior studies focused on the most-studied DMEs and transporters, such as cytochrome P450s (CYPs) and uridine 5'-diphospho-glucuronosyltransferases (UGTs), leaving many other important enzymes undetermined (e.g. hydrolases, sulfotransferases [SULTs], N-acetyltransferases [NATs], glutathione S-transferases [GSTs], and house-keeping proteins). In practice, HLM and HLS9 are often selected arbitrarily by investigators for *in vitro* metabolism studies in part due to the lack of a quantitative understanding of the differences between the proteomes of these two sample types (Plant, 2004). Moreover, without knowing the differences, it is difficult for researchers to reconcile the results obtained from these distinct *in vitro* models. Therefore, a comparative proteomics analysis of HLM and HLS9 is needed to inform the selection of one *in vitro* model over another and to improve the interpretation of results generated from the two hepatic preparations.

With the rapid advance of mass spectrometry-based proteomics, absolute quantitative proteomics (APQ) has been increasingly used in both basic and clinical investigations to absolutely quantify protein levels in various biological samples(Prasad et al., 2019). We recently developed a novel label-free global APQ method, named DIA-TPA, using data-independent acquisition (DIA) and the total protein approach (TPA) algorithm (He et al., 2019). Compared to other

labeling global APQ proteomics approaches, such as metabolic labeling (e.g. stable isotope labeling using amino acid in cell culture, SILAC) (Hanke et al., 2008) and chemical labeling (e.g. isobaric tags for relative and absolute quantitation, iTRAQ) (Wiese et al., 2007), the DIA-TPA method offers an efficient and cost-effective option for absolutely measuring the whole proteome. Additionally, unlike most label-free APQ methods (Ishihama et al., 2005; Silva et al., 2006; Braisted et al., 2008; Schwanhausser et al., 2011; Schwanhausser et al., 2013; Shin et al., 2013; Wisniewski et al., 2014) that use MS1 signals from data-dependent acquisition (DDA) for quantification, the DIA-TPA method relies on the MS2 signals from DIA, which effectively avoids the often-seen bias of DDA for the most intense precursors (Huang et al., 2015). Moreover, DIA-TPA employs a novel algorithm to allocate the signals of shared peptides, allowing for accurate quantification of protein isoforms. As DMEs and transporters often consist of multiple isoforms with significant portions of shared amino acid sequence, the DIA-TPA method is ideally suited for the absolute quantification of the proteomes of HLM and HLS9.

In the present study, we performed a global absolute quantitative proteomics analysis of 102 individual HLS9 samples and the corresponding HLM preparations using the DIA-TPA method. The study revealed the proteome profiles of paired HLM and HLS9 samples and compared the levels and interindividual variabilities of DME and transporter protein concentrations between HLM and HLS9. The findings will allow us to make more informed decisions when choosing between HLM and HLS9 for an *in vitro* drug

metabolism study, and will also lead to better understanding of the results from incubation studies involving HLM and HLS9.

Materials and Methods

Materials

Urea, ammonium bicarbonate (NH_4HCO_3), dl-dithiothreitol (DTT), trifluoroacetic acid (TFA), acetone and water with 0.1% formic acid (LC-MS reagent) were purchased from Fisher Scientific Co. (Pittsburgh, PA). Acetonitrile with 0.1% formic acid (LC-MS reagent) was from JT Baker Chemical Co. (Phillipsburg, NJ). Iodoacetamide (IAA) was the product of Acros Organics (Morris Plains, NJ). TPCK-treated trypsin was obtained from Worthington Biochemical Corporation (Freehold, NJ). Lysyl endopeptidase (Lys-C) was purchased from Wako Chemicals (Richmond, VA). Water Oasis HLB columns were obtained from Waters Corporation (Milford, MA). Synthetic iRT standards solution was produced by Biognosys AG (Cambridge, MA). Pierce™ BCA protein assay kit and phosphate-buffered saline (PBS) were from Thermo Fisher Scientific (Waltham, MA). All other chemicals and reagents were of analytical grade and commercially available.

A total of 102 normal individual human liver samples were obtained from XenoTech LLC (Kansas City, KS) and the Cooperative Human Tissue Network (CHTN, Columbus, OH). All the tissues were snap frozen in liquid nitrogen and stored at -80°C until use. The donors consisted of 46 males and 56 females with ages ranging from 0.75 to 83 years, and included 95 Caucasians, five African-Americans, one Hispanics, and one classified as ‘others’. The use of these liver

samples was approved by the IRB.

Preparation of HLS9 and HLM

HLS9 and HLM were prepared from the 102 individual human liver samples using a method described in previous publications (Wang et al., 2016; Shi et al., 2018). Briefly, ~200 mg individual liver tissues were homogenized in 0.5 ml PBS in 1.5 ml microcentrifuge tubes on ice using an automatic pestle (VWR International LLC, Chicago). The homogenates were then centrifuged twice at 9,000 ×*g* for 20 min at 4 °C to remove the fat-containing top layers. The supernatant was collected as HLS9 and transferred to a clean tube. To prepare HLM, HLS9 was further centrifuged at 300,000 ×g for 20 min at 4 °C using an Optima™ MAX ultracentrifuge with a MLA-80 rotor (Beckman Coulter, Indianapolis, IN)(Braner et al., 2019). The pellet (HLM) formed after the centrifugation was then re-suspended in PBS and transferred to a clean tube for storage. Total protein concentrations of HLS9 and HLM samples were determined using a Pierce™ BCA protein assay kit. Pooled HLS9 and HLM samples were prepared by mixing protein content-normalized aliquots of the given sample type from all individuals. Samples were kept on ice or at 4 °C during the entire preparation process, and the prepared HLS9 and HLM samples were stored at -80 °C until use. The recovery factors for HLS9 (s9 protein per gram liver, S9PPGL) and HLM (microsomal protein per gram liver, MPPGL) (Wilson et al., 2003) were determined to be 107.3 ± 28.1 mg/g liver and 18.8 ± 6.4 mg/g liver. Respectively.

Proteomics sample preparation

HLS9 and HLM samples were prepared for proteomics analysis according to a previously published method with some minor modifications (Shi et al., 2018). In brief, 80 µg HLS9 or HLM protein aliquot was mixed with 0.2 µg bovine serum albumin (BSA) in a 1.5 ml microcentrifuge tube. One ml (50-fold volume) of pre-cooled acetone was added to the mixture and stored at -20 °C overnight to precipitate proteins. The samples were centrifuged at 17,000 ×*g* for 15 min at 4 °C. Following centrifugation, the supernatant was removed, and the pellet air-dried at room temperature for 5 min. The dried pellet was then re-suspended in 100 µl freshly prepared 4 mM DTT in 8 M urea solution with 100 mM NH_4HCO_3, followed by vortexing and sonication to ensure complete dissolution of proteins. The samples were then incubated at 37 °C for 45 min for reduction. After cooling to room temperature, 100 µl freshly prepared 20 mM IAA in 8 M urea solution with 100 mM NH_4HCO_3 was added and mixed, followed by incubation at room temperature in the dark for 30 min. Subsequently, the urea concentration was adjusted to 6 M by the addition of 56.6 µl 50 mM NH_4HCO_3. For protein digestion, samples were first incubated with Lys-C (protein:Lys-C = 100:1) in an orbital incubator shaker at 220 rpm, 37 °C for 6 hours. Next, the urea concentration was further adjusted to 1.6 M by adding 733 µl 50 mM NH_4HCO_3, followed by an overnight incubation with TPCK-treated trypsin (protein:trypsin = 50:1) at 220 rpm and 37 °C . Digestion was terminated by adding 1 µl TFA. The digested peptides were extracted and cleaned using Waters Oasis HLB columns according to the manufacturer's instructions. The eluted peptides were dried using a SpeedVac SPD1010 vacuum concentrator (Thermo Scientific, Hudson, NH, USA) and re-

suspended in 3% acetonitrile solution with 0.1% formic acid. The re-constituted peptide solution was then centrifuged at 17,000 ×*g* for 10 min at 4 °C. Half of the supernatant was transferred to a clean autosampler vial and mixed with 1 µl iRT standard solution for LC-M/MS analysis.

LC-MS/MS-based proteomics analysis

The LC-MS/MS system consisted of a tandem quadrupole time-of-flight mass spectrometer (AB/Sciex TripleTOF5600 plus, Framingham, MA) and an Eksigent 2D plus LC system (Eksigent Technologies, Dublin, CA). The analysis was conducted using a previously published method with some modifications (Shi et al., 2018). Briefly, the mobile phase was composed of water with 0.1% formic acid (A) and acetonitrile containing 0.1% formic acid (B). Peptides were separated through a trap-elute configuration, including a trapping column (ChromXP C18-CL, 120 Å, 5 µm, 0.3 mm catridge, Eksigent Technologies, Dublin, CA) and an analytical column (ChromXP C18-CL, 120 Å, 150 × 0.3mm, 5 µm, Eksigent Technologies, Dublin, CA). Six µg of digested protein was injected, trapped, and cleaned on the trapping column with mobile phase A at a flow rate of 10 µl/min for 3 min, and then was separated on the analytical column with a gradient elution at a flow rate of 5 µl/min (**Supplemental Table 1**). Each sample was followed by a blank sample injection to minimize carryover. Ionization was achieved via positive ion mode with an ion spray voltage at 5,500 V and source temperature at 280 °C.

DIA and DIA-TPA protein quantification

HLM and HLS9 samples were analyzed using a data-independent acquisition

(DIA) method (Shi et al., 2018), which comprised of a 250 ms TOF-MS scan from 400 to 1250 Da, followed by MS/MS scans from 100 to 1500 Da performed on all precursors in a cyclic manner using a 100-variable isolation window scheme. The accumulation time was 25 ms per isolation window resulting in a total cycle time of 2.8 s. Spectronaut™ Pulsar software (version 11.0, Biognosys AG, Schlieren, Switzerland) and its own standard reference spectral library "Human-Liver (fractionated)" were used to process the DIA data and obtain MS2 signal intensities for label-free absolute protein quantification using the previously published DIA-TPA method (He et al., 2019). Quantification was performed only for proteins with at least two unique peptides. Comprehensive lists of human hydrolases and transferases were downloaded from UniProtKB/Swiss-Prot enzyme class 2 and 3, respectively.

Statistical analysis

GraphPad Prism® version 6.02 (GraphPad Software, San Diego, CA) was used for statistical analysis and generating graphs. The Venn diagram was generated using an online tool developed by the Van de Peer lab (Bioinformatics & Evolutionary Genomics) http://bioinformatics.psb.ugent.be/webtools/Venn/. Since a considerable proportion of proteins had concentrations that did not follow the Gaussian distribution, nonparametric statistical analysis was performed. Specifically, two-tailed nonparametric Spearman correlation and paired nonparametric t-test (Wilcoxon matched-pairs signed rank test) were used to assess the correlations and differences between different features, respectively. A P-value ≤ 0.05 was considered statistically significant.

Results

Proteome profile comparison between HLM and HLS9

A total of 3,137 proteins were absolutely quantified in the HLM and HLS9 samples. Among these, 3,087 proteins (98.4%) were shared by the two hepatic preparations, and 48 and two proteins were only quantified in HLM and HLS9, respectively. In general, mean protein concentrations were highly correlated between HLM and HLS9 samples (**Figure 1**, R=0.87, P<0.0001). Regarding correlations of specific proteins between HLM and HLS9 samples across individuals, 690 out of 3,137 proteins were not correlated (R<0.20, P>0.05); 701 proteins showed a very weak correlation (0.20≤R<0.40, P<0.05); 771 proteins exhibited a weak correlation (0.40≤R<0.60, P<0.05); 742 proteins were in moderately strong correlation (0.60≤R<0.80, P<0.05); and 233 proteins were in very strong correlation (0.80≤R<1.00, P<0.05) (**Figure 2A**). With respect to differences of protein concentrations between HLM and HLS9 samples, the concentrations of 1,834 proteins were higher in HLM than in HLS9 with HLM to HLS9 ratios ranging from 1.11 to 826.98 (P<0.05); meanwhile, 814 proteins were found to be more concentrated in HLS9 than HLM with HLS9 to HLM ratios from 1.42 to 75.58 (P<0.05) (**Figure 2B**).

Phase I DMEs in HLM and HLS9

CYPs

Twenty-five CYPs were quantitated in both sample types, constituting 1.23% and 0.40% of the HLM and HLS9 proteomes, respectively. As expected, CYP protein

concentrations were higher in HLM than HLS9, with HLM to HLS9 concentration ratios ($CYP_{(i)\_HLM}/CYP_{(i)\_HLS9}$) ranging from 2.68 to 50.64 (**Supplemental Table 2**). Additionally, protein levels of the 25 CYP enzymes varied significantly. CYP2A6 was the most abundant CYP enzyme in both hepatic preparations, while CYP17A1 was the least abundant in both (**Figure 3A**). Meanwhile, protein concentrations between CYP2A6 and CYP17A1 respectively differed 5,474-fold and 5,582-fold in HLM and HLS9 samples. Significant interindividual variability in CYP concentrations was observed across the 102 individual HLM and HLS9 samples, with coefficients of variation (CVs) ranging from 41.43% to 1009.95%. CYP17A1, the least abundant CYP, was found to be the most variable, with CVs of 776.11% and 1009.95% in HLM and HLS9, respectively. The substantial interindividual variability in CYP17A1 concentrations is likely in part due to less accuracy in quantifying low abundant proteins. CYP4F2 was the least variable CYP enzyme in both HLM and HLS9 with identical CVs of 41.43% (**Supplemental Table 2**).

Of human CYPs, only about a dozen are involved in drug metabolism (Zanger and Schwab, 2013). The concentrations of these drug metabolism-related CYPs ranked as follows: in HLM: CYP2A6 > CYP2C8 > CYP2C9 > CYP2E1 > CYP3A4 > CYP2C19 > CYP1A2 > CYP2D6 > CYP3A5 > CYP2B6 > CYP2J2; and in HLS9: CYP2A6 > CYP2C9 > CYP2C8 > CYP2C19 > CYP2E1 > CYP3A4 > CYP1A2 > CYP2D6 > CYP3A5 > CYP2B6 > CYP2J2 (**Figure 3A**). Moreover, the protein levels of these CYPs were highly correlated between HLM and HLS9 samples (**Supplemental Figure 1**, R: 0.72 - 0.93, P<0.0001). The HLM to HLS9

ratios of these CYPs were within the range of 2.68 and 3.64 (**Supplemental Table 2**).

Hydrolases

A total of 308 hydrolases were absolutely quantitated in the HLM and HLS9 samples, of which 36 have been reported to be involved in drug metabolism(Testa and Kramer, 2007). Among these, 11 hydrolases had significantly lower mean concentrations in HLM than HLS9 ($Hydrolase_{(i)\_HLM}/Hydrolase_{(i)\_HLS9}$: 0.22-0.85, $P<0.05$), while the remaining 22 hydrolases were significantly more concentrated in HLM ($Hydrolase_{(i)\_HLM}/Hydrolase_{(i)\_HLS9}$: 1.22-26.57, $P<0.05$). Carboxylesterase1 (CES1) was the most abundant hydrolase in both HLM and HLS9, with concentrations of 286.73 ± 100.28 pmol/mg protein and 205.33 ± 70.51 pmol/mg protein, respectively. Interestingly, two epoxide hydrolases (epoxide hydrolase1, EPHX1 and epoxide hydrolase2, EPHX2) were distributed between HLM and HLS9 in accordance with their subcellular locations. Specifically, the microsomal epoxide hydrolase (EPHX1) was significantly higher in HLM than HLS9 ($EPHX1_{\_HLM}$/ $EPHX1_{\_HLS9}$ = 2.79), whereas the cytosolic epoxide hydrolase (EPHX2) had a HLM to HLS9 ratio of 0.57 (**Figure 3B** and **Supplemental Table 3**).

Phase II DMEs in HLM and HLS9

UGTs

The study quantified 13 and 12 UGTs in HLM and HLS9 samples, respectively. In HLM, the protein concentrations ranged from 6.51 fmol/mg to 18.28 pmol/mg protein, versus 2.75 fmol/mg to 5.81 pmol/mg protein in HLS9 (**Figure 3C**). All quantified UGTs were higher in HLM than in HLS9 with fold differences ranging from 2.75 to 60.21 ($UGT_{(i)\_HLM}/UGT_{(i)\_HLS9}$). The most abundant UTGs were UGT2B7 in HLM (18.28 ± 9.68 pmol/mg protein) and UGT2B15 in HLS9 (5.92 ± 2.79 pmol/mg protein). UGT3A1 had the lowest concentration in HLM (6.51 ± 0.24 fmol/mg protein), and was not detected in HLS9. Similar to other DMEs, significant interindividual variation in UGT concentrations was observed across the 102 individual human liver samples. UGT2B15 exhibited the smallest interindividual variation in both HLM (CV = 47.84%) and HLS9 (CV = 47.09%), whereas those with the greatest variation were UGT3A1 in HLM (CV = 362.19%) and UGT2A3 in HLS9 (CV = 710.72%) (**Supplemental Table 4**).

Other transferases

In addition to UGTs, 24 out of the 295 transferases quantitated in this study are also involved in the phase II reactions of various medications. Those transferases include thiopurine S-methyl transferase (TPMT), catechol-O-methyltransferase (COMT), and enzymes from the glutathione S-transferase (GST), sufotransferase (SULT), and N-acetyltransferase (NAT) families. The majority of the transferases had significantly higher concentrations in HLS9 than in HLM ($transferase_{(i)\_HLM}/transferase_{(i)\_HLS9}$: 0.15-0.85, $P<0.05$), except for NAT8 and the microsomal glutathione S-transferases (MGST1, 2, and 3) with the ratios ranging from 4.21 to 14.15 (**Figure 3D** and **Supplemental Table 5**).

Transporter proteins in HLM and HLS9

We quantified protein concentrations of 51 transporters, including ten ATP-binding cassette (ABC) transporters and 41 transporters from the solute carrier family (SLC). All transporters were more abundant in HLM than in HLS9. ABCB4 and six SLC transporters (SLC1A4, SLC16A1, SLC30A7, SLC39A7, SLC44A1 and SLCO1B3) were only detected in HLM, at relatively low levels. For the transporters detected in both HLM and HLS9, the differences in protein concentration between sample types ranged from 1.16- to 826.98-fold (**Supplemental Table 6**). ABCD3 was the most abundant ABC transporter in both HLM and HLS9 samples. The protein concentration of ABCB1/MDR1 (P-glycoprotein, P-gp), the most studied efflux drug transporter, ranked ninth among the ten ABC transporters in both HLM and HLS9 samples.

Variability of commonly used housekeeping proteins

To evaluate the variability of commonly used housekeeping proteins in human livers, we determined the protein concentrations of β-actin (ACTB), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and β-tubulin (TUBB) in the 102 individual HLM and HLS9 samples. β-actin showed comparable protein concentrations in HLM (3.32 ± 2.41 pmol/mg protein) and HLS9 (2.99 ± 1.48 pmol/mg protein); GAPDH levels were slightly higher in HLS9 (217.26 ± 38.01 pmol/mg protein) than in HLM (187.56 ± 47.20 pmol/mg protein), while β-tubulin was more abundant in HLM (10.13 ± 5.54 pmol/mg protein) compared to HLS9 (4.31 ± 2.52 pmol/mg protein). The interindividual variability of these housekeeping proteins was as follows: in HLM, β-actin (CV: 72.52%) > β-tubulin

(CV: 54.68%) > GAPDH (CV: 25.16%); and in HLS9, β-tubulin (CV: 58.44%) > β-actin (CV: 49.50%) > GAPDH (CV: 17.50%). To identity other potential housekeeping proteins for the normalization of protein quantification in the liver, we also evaluated the variability of protein concentrations across the 102 individual HLM and HLS9 samples and identified five proteins with the smallest CVs (13.32%-20.76%) (**Table 1**). These proteins could potentially serve as new internal controls for the measurement of hepatic protein expression (e.g. in Western blots).

Organelle and cytosolic protein markers

The concentrations of membrane marker proteins for the endoplasmic reticulum (ER), mitochondria, proteasome, ribosome, Golgi apparatus and plasma membrane were all higher in HLM than in HLS9 (HLM/HLS9 > 2.00, $P<0.05$). Among these organelle membrane marker proteins, the plasma membrane signature protein sodium/potassium-transporting ATPase subunit alpha-1 (ATP1A1) showed the highest HLM-to-HLS9 ratio (7.70), followed by mitochondrial protein (cytochrome c oxidase subunit 4 isoform 1, COX4I1) (3.76), ribosome specific protein (60S ribosomal protein L7a, RPL7A) (3.62), ER marker protein (epoxide hydrolase 1, EPHX1) (2.79), proteasome marker protein (26S proteasome non-ATPase regulatory subunit 1, PSMD1) (2.34), and Golgi apparatus protein (formimidoyltransferase cyclodeaminase, FTCD) (2.24) (**Table 2**). As a comparison, the two cytosolic proteins thymosin beta-4 (TMSB4X) and aldo-keto reductase family 1 member A1 (AKR1A1) had concentrations that were respectively 7.68- and 4.28-fold higher in HLS9 than in HLM (**Table 2**).

Sex effect on protein levels in HLS9 and HLM

The concentrations of all quantified proteins were compared between the male (n=46) and female (n=56) liver samples. A total of 36 HLM proteins and 10 HLS9 proteins were significantly different between males and females (**Figure 4)**. Among the 36 HLM proteins, 24 proteins exhibited significantly higher levels in males than in females, whereas the rest 12 proteins were found to have lower concentrations in males, with the male to female ratios protein concentrations ranging from 0.16 to 5.18. For the 10 HLS9 proteins, the concentrations of three proteins were higher in males, while the rest seven proteins exhibited higher levels in females. The male to female ratios of the concentrations of these HLS9 proteins ranged from 0.14 to 4.63 (**Supplemental Table 7**). Among these sex-associated proteins, UGT2B17 was found to be 2.28-fold higher in males than females ($P < 0.05$), which was consistent with the previous findings (Gallagher et al., 2010).

Protein-protein correlations in HLM and HLS9 samples

To characterize the co-regulation patterns of the proteins involved in drug metabolism and disposition, we analyzed the protein-protein correlations among DMEs and transporters in HLM and HLS9 (**Figure 5**). Strong protein-protein correlations were observed within several DME and transporter groups in both HLM and HLS9 samples. Nine transporters in the SLC25 family, including SLC25A1, SLC25A10, SLC25A11, SLC25A13, SLC25A15, SLC25A20, SLC25A4, SLC25A5, and SLC25A6, showed strong correlations with each other, with spearman's R ranging from 0.71 to 0.98 in HLM and 0.41-0.90 in HLS9. In

addition, four UGT1As (UGT1A4, UGT1A6, UGT1A7, and UGT1A9) and five UGT2Bs (UGT2B4, UGT2B7, UGT2B10, UGT2B15, and UGT2B17) were also strongly correlated within the corresponding sub-families (R: 0.72-0.97 in HLM; R: 0.70-0.90 in HLS9). Moreover, intra-family protein-protein correlations were found for the enzymes within the CES (CES1 and CES2, R = 0.62) and CYP2 families (CYP2A6, CYP2B6, CYP2C8, CYP2C9, and CYP2C19, R =0.50-0.78) in HLM. Interestingly, significant inter-family correlations were also observed for the CES and CYP2 enzymes across the two families in HLM (R: 0.51-0.70). Similarly, the CYP2 enzymes in HLS9 were significantly correlated (R: 0.50-0.77). However, neither the CESs enzymes nor the enzymes across the CESs and CYP2 families were found to be significantly correlated in HLS9 (**Figure 5**).

Discussion

Although HLM and HLS9 have been widely used for the study of drug metabolism, our knowledge of their protein constitutions, especially absolute protein quantities, remains limited. Recently, in conjunction with advances in LC-MS/MS-based quantitative proteomics, there is an increasing interest in studying the protein profiles of HLM and HLS9. To date, most proteomics studies have been conducted on HLM and focused on several major DMEs, such as CYPs (Kawakami et al., 2011; Liu et al., 2014) and UGTs (Achour et al., 2017b), and drug transporters (Ohtsuki et al., 2012b; Achour et al., 2014b; Achour et al., 2017a; Couto et al., 2019); and only a few investigations have included both HLM and HLS9 samples. For example, Fallon et al. determined the concentrations of

14 UGT1As and UGT2Bs in individual HLM and HLS9 samples using a targeted proteomics method (Fallon et al., 2013). Recently, a label-free APQ method, named DIA-TPA, was developed for absolute global proteome quantification (He et al., 2019). This method has been validated by comparing its quantification results of several DMEs in HLS9 with the reference values reported from previous studies based upon labeled internal standard-based absolute protein quantification (He et al., 2019). Another global proteomics study compared the differences of hepatic proteins among human, rat, and mouse using pooled liver microsomes and S9 fractions and identified 1516 and 1570 proteins in HLM and HLS9, respectively, but no quantitative analysis was performed (Golizeh et al., 2015a; Golizeh et al., 2015b). The lack of a comprehensive comparative quantitative analysis of the HLM and HLS9 proteomes contributes to uncertainty when choosing one hepatic preparation over another and leads to difficulties in interpreting the data obtained from these two different *in vitro* models. To fill this knowledge gap, we conducted a global quantitative proteomics analysis of 102 HLS9 and their corresponding HLM samples and compared the protein profiles between these two preparations. We found that HLM and HLS9 shared 3,087 proteins out of a total of 3,137 quantified proteins. As expected, proteins located in the cytosol or associated with the cytoskeleton were more concentrated in HLS9 relative to HLM. In contrast, membrane proteins, such as CYPs, UGTs, and transporters, exhibited higher concentrations in HLM than HLS9 (**Table 2 and Supplemental Table 2, 4, and 6**).

Significant interindividual variability in protein expressions has been well documented in human livers (Wilkinson, 2005; Wortham et al., 2007), and HLM and HLS9 have been widely used for the study of interindividual variability in drug metabolism (Shimada et al., 1994; Wang et al., 2016). Nevertheless, it is unclear whether the extra steps involved in preparing HLM from HLS9 would introduce additional variation on top of interindividual biological variability in the liver. Using the 102 individual HLS9 and their corresponding HLM samples, we examined the interindividual variability of proteins in both preparations. Among the 3,087 proteins shared by HLM and HLS9, 1,205 and 1,882 showed higher CVs in HLM and HLS9, respectively, suggesting that HLM sample preparation procedures did not introduce additional variation in our experiment. Interestingly, significant negative correlations were observed between CVs and the mean protein concentrations in both HLM (**Supplemental Figure 2A**, $R = -0.81$, $P < 0.0001$) and HLS9 (**Supplemental Figure 2B**, $R = -0.88$, $P < 0.0001$) samples. Therefore, part of the variability may be ascribable to proteomics assay errors, which tend to have a more profound impact on the quantification of low abundance proteins (e.g. CYP17A1 and UGT3A1). Demographic factors, such as sex, could contribute to the interindividual variability in the levels of some proteins. By comparing the liver proteomes between males and females, we found that UGT2B17 concentrations were significantly higher in males than females in HLM (**Supplemental Table 7,** male/female =2.28, $P<0.05$). This finding was in agreement with the previous report that the protein expression and activity of UGT2B17 were about 4-fold and 1.5-3 times higher, respectively, in

males than females (Gallagher et al., 2010). However, the sex difference in UGT2B17 expression was not observed in HLS9, which is likely caused by the inaccurate protein quantification associated with the low abundance of UGT2B17 in HLS9.

With the 102 matched HLS9 and HLM samples, we were able to identify a significant positive correlation in protein concentrations between the two preparations (**Figure 1**, R=0.87, P<0.0001). Of note, the correlation was relatively weak for proteins with low concentrations, which could be in part due to the less accurate quantifications of those low-abundance proteins. Besides the correlations of proteins between the two liver preparations, we also analyzed the protein-protein correlation within each liver preparation to explore protein expression co-regulations in human livers (Wang et al., 2017). SLC transporters, CYP2, and UGT2B proteins showed significant protein-protein correlations within their own family groups in both HLM and HLS9, indicating that the proteins within these families might share certain common regulatory factors, e.g. nuclear receptors (Chen et al., 2012) and miRNAs (Ikemura et al., 2014). Interestingly, in addition to being highly correlated to each other, CES1 and CES2 were also significantly correlated with the CYP2 isoenzymes including CYP2A6, CYP2B6, CYP2C8, CYP2C9, and CYP2C19 in HLM.

To date, absolute protein quantification of HLM and HLS9 has been mostly performed using multiple-reaction monitoring (MRM)-based targeted proteomics methods with stable isotope internal standards, such as stable isotope-labeled with synthetic peptides (AQUA) and quantification concatamers (QconCAT)

(Kawakami et al., 2011; Ohtsuki et al., 2012b; Fallon et al., 2013; Achour et al., 2014b; Achour et al., 2017b). In comparison with label-free absolute quantitative proteomics, those stable isotope internal standards-based targeted methods are often more expensive and time-consuming. More importantly, the targeted methods are limited in the number of proteins that can be simultaneously quantified (Achour et al., 2014a; Michaels and Wang, 2014; Prasad et al., 2019). Recently, a global label-free proteomics study was conducted to quantify protein concentrations in HLM, with a focus on DMEs (Achour et al., 2017a). Distinct from earlier studies, the present investigation employed a newly developed label-free absolute global quantitative proteomics method, namely DIA-TPA, to compare HLM and HLS9 proteomes using a large set of human liver samples. The DIA-TPA method allows for absolute quantification at the proteome level, and its unique algorithm is particularly suited for the quantification of protein isoforms with highly similar amino acid sequence, such as CYPs and UGTs (He et al., 2019). Indeed, the present study quantified more CYPs and UGTs than previous label-free absolute quantitative methods (Achour et al., 2017a; Couto et al., 2019). As an example, several CYP4F isoenzymes including CYP4F3 and CYP4F8 were successfully quantitated by the DIA-TPA method in current study for the first time.

Recently, protein concentrations of CYPs and UGTs in HLM have been intensively studied using MS-based proteomics (Kawakami et al., 2011; Ohtsuki et al., 2012b; Fallon et al., 2013; Achour et al., 2014b; Liu et al., 2014; Prasad et al., 2019); however, a comparative analysis of these enzymes in HLM and HLS9 remains wanting. In the current study, we revealed significant differences in CYP and UGT protein concentrations between the two preparations, as well as

DMD Fast Forward. Published on November 7, 2019 as DOI: 10.1124/dmd.119.089235
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marked interindividual variability among individuals. The concentrations of CYPs and UGTs in HLM were over two times higher than in HLS9 (**Supplemental Table 2 and 4**), which is consistent with both CYPs and UGTs being membrane-bound proteins. Given the very low levels of UGTs in HLS9 (UGT_{HLM}/UGT_{HLS9}: 2.75-60.21), HLM appears to be preferable for *in vitro* study of UGTs. For those drug metabolism-related CYPs, protein concentrations correlated well between the matched HLS9 and HLM samples (R: 0.72-0.93, P<0.0001, **Supplemental Figure 1**). We also found that the concentrations of CYPs and UGTs in HLM were comparable to the values reported by previous isotope labeling-based proteomics studies (Kawakami et al., 2011; Ohtsuki et al., 2012a; Ohtsuki et al., 2012b; Achour et al., 2014b; Groer et al., 2014; Michaels and Wang, 2014). Moreover, to further validate the protein quantification results, enzymatic activities were determined for CYP2D6 in HLS9 and UGT2B15 in HLM (**Supplemental Figure 4**). We observed significant correlations between the enzymatic activities and the protein levels (**Supplemental Figure 4**, R: CYP2D6: 0.6942 and UGT2B15: 0.6077). The strength of the correlation coefficients is comparable to that reported in previous label-based targeted proteomics studies, further supporting the validity of our label-free global APQ assay (Ohtsuki et al., 2012b; Achour et al., 2014b; Achour et al., 2017b). In general, the CYPs relevant to drug metabolism were more abundant in HLM than HLS9. However, given that the differences were relatively small, and the protein levels of these CYPs were highly correlated between the two liver preparations, either HLM or HLS9 appears appropriate for the study of these CYP enzymes in the liver.

In addition to CYPs and UGTs, other phase I and phase II enzymes such as hydrolases and other transferases also play vital roles in catalyzing the metabolism of many drugs and toxins. However, our knowledge on the protein profiles of these enzymes in HLM and HLS9 is limited compared to CYPs and UGTs. In this study, we quantified 36 hydrolases and 24 transferases in addition to UGTs in HLM and HLS9 samples (**Supplemental Table 3** and **5**). In general,

DMD Fast Forward. Published on November 7, 2019 as DOI: 10.1124/dmd.119.089235
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hydrolases were the most abundant enzyme group among the four classes of DMEs (i.e. CYPs, hydrolases, UGTs, and transferases), and constituted 76.17% and 66.46% of DMEs in HLM and HLS9, respectively (**Supplemental Figure 3**). CES1 was the most abundant enzyme, accounting for 15.75% and 14.31% of all DME proteins and 1.81% and 1.29% of the whole proteomes in HLM and HLS9, respectively. As expected, the hydrolases with significantly higher HLM concentrations ($Concentration_{HLM}/Concentration_{HLS9}$ >2.0, P<0.05) were primarily located at membranes. Interestingly, although PONs are known to be mostly excreted to the systemic circulation (Fukami and Yokoi, 2012), PON1, PON2 and PON3 exhibited significantly higher concentrations in HLM than HLS9. Consistent with protein subcellular locations, five hydrolases located in cytosol or nucleus (CMBL, ABHD10, ESD, ABHD14B and PAFAH1B2) showed significantly higher concentrations in HLS9 than HLM ($Concentration_{HLM}/Concentration_{HLS9}$ < 0.50, P<0.05), and the concentrations of hydrolases associated with membranes or the ER appeared to be significantly higher in HLM than HLS9 ($Concentration_{HLM}/Concentration_{HLS9}$: 2.15-3.11, P<0.05). In contrast to other classes of DMEs, the majority of transferases including the GSTA, GSTK, GSTM, GSTO, and SULT families were more abundant in HLS9 than HLM ($Concentration_{HLM}/Concentration_{HLS9}$: 0.15-0.85, P<0.05), with the MGST family and NAT8 being only the transferases that had higher concentrations in HLM ($Concentration_{HLM}/Concentration_{HLS9}$: 4.21-14.15, P<0.05).

Transporters in human livers are another major focus in drug disposition research and have also been recognized as therapeutic targets for drug

development (Leonard et al., 2002; Faber et al., 2003; Lin et al., 2015; Couto et al., 2019). In humans, ABC and SLC are two main transporter families, respectively involved in the efflux and uptake of small molecules. In this study, a total of ten ABC transporters and 41 SLC transporters were quantified in HLM, all at relatively higher concentrations than those in HLS9. Of these, ABCB4 and six SLC proteins (SLC1A4, SLC16A1, SLC30A7, SLC39A7, SLC44A1, and SLCO1B3) were only detected in HLM, not in HLS9. Thus, HLM is more suitable for transporter studies compared to HLS9 due to the extremely low concentrations of most transporters in HLS9. However, it is noted that better coverage of transporters can be achieved if utilizing a sample preparation protocol optimized for transporter protein extraction.

Microsomes are often regarded as cellular fractions with high ER membrane content (Fouts, 1961). However, information is scarce concerning the presence of other cell organelles in HLM and HLS9 preparations. This study determined the concentrations of representative protein markers for several organelle membranes and cytosol. We found that apart from the ER marker EPHX1, protein markers for the plasma membrane and many other organelles, i.e. ribosome (RPL7A), proteasome (PSMD1), mitochondria (COX4I1), and Golgi body (FTCD) are also more concentrated in HLM, whereas the cytosol protein marker AKR1A1 and the cytoskeleton marker TMSB4X showed higher concentrations in HLS9. These observations are in accordance with the fact that HLM is mainly composed of the membranes of hepatic plasma and various organelles, while HLS9 contains both HLM and cytosol (**Table 2**).

For decades, housekeeping proteins have been routinely used as internal controls to normalize protein-loading in Western blot experiments (Sullivan-Gunn et al., 2011) with the assumption that their expression levels are constant across samples. However, it is increasingly reported that housekeeping protein expression can be affected by many physiological and environmental factors (Ferguson et al., 2005; Rubie et al., 2005; Congiu et al., 2011), which could lead to inaccurate protein expression measurements (Sullivan-Gunn et al., 2011; Li and Shen, 2013). Hence, we analyzed the concentrations of the most widely used housekeeping proteins, including β-actin, GAPDH and β-tublin, in the 102 matched HLM and HLS9 samples. In both HLM and HLS9 samples, GAPDH was the most abundant housekeeping protein (ranked 22 and 17 among all proteins in HLM and HLS9, respectively), and had the least interindividual variability (CV: 25.17% in HLM and 17.50% in HLS9). To search for new housekeeping proteins suitable for use as internal controls in Western blot assays of human liver samples, we identified five proteins with the smallest variability among the 102 individual HLM (CV: 17.53-20.76%) and HLS9 (CV: 13.32-16.77%) samples (**Table 1**). Further validation is warranted to evaluate the applicability of these proteins as new internal controls for Western blot experiments.

In summary, the present study compared the proteome profiles of the two most commonly used hepatic preparations, HLM and HLS9, and determined the concentrations and interindividual variability of many DMEs and transporters as well as several housekeeping and subcellular marker proteins in a large set of matched HLM and HLS9 samples. The study revealed marked differences in

protein concentrations between HLM and HLS9. The knowledge generated from the study may help us better interpret data obtained from the two different hepatic preparations and may assist in *in vitro*-*in vivo* extrapolations. The data also allow us to make more informed decisions when choosing between HLM and HLS9 for an *in vitro* drug metabolism study.

Authorship Contributions

Participated in research design: Wang, Shi, Zhu

Conducted experiments: Wang, Shi, Li

Performed data analysis: Wang, He, Shi, Zhu

Contributed new reagents or analytic tools: Li

Wrote and contributed to the writing of the manuscript: Wang, Zhu

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Footnotes

This work was partially supported by the National Institutes of Health National Heart, Lung, and Blood Institute [Grant R01HL126969, Hao-Jie Zhu], and the Michigan Institute of Clinical Health Research (MICHR) Pre-K Award [Grant UL1TR002240, Xinwen Wang].

Legends for Figures:

Figure 1. Correlation between the mean protein abundances in the 102 HLM and the matched HLS9 samples. Absolute protein concentrations were obtained using the label-free DIA-TPA method. Spearman's correlation analysis was performed and $P<0.05$ was considered as statistically significant.

Figure 2. Distributions of the Spearman's R values of the protein concentration correlations between HLM and HLS9 (A) and the HLM to HLS9 ratios of mean protein concentrations (B). 50 proteins that were only quantified in one type of samples were removed from the analysis.

Figure 3. Mean protein concentrations of CYPs (A), hydrolases (B), UGTs (C), and other transferases (D) in the 102 matched individual HLM and HLS9 samples. Black and grey bars represent protein concentrations in HLM and HLS9, respectively, and error bars represent standard deviation.

Figure 4. The differences in the liver proteomes between males and females in HLM (A) and HLS9 (B). The x-axis is the $\log_2$ protein concentration ratios of males to females, with the two vertical dotted lines indicating a two-fold difference between males and females. The y-axis indicates the $-\log_{10}$ (P-value) with a horizontal dotted line at $P=0.05$, and the higher the y is, more significant the difference would be. The red and blue dots represent the proteins being significantly higher in males and females, respectively.

Figure 5. The heatmap of protein-protein correlation analysis in HLM (A) and HLS9 (B). Red and blue shaded boxes indicate, respectively, the extents of positive and negative correlations; white boxes indicate no correlation.

DMD Fast Forward. Published on November 7, 2019 as DOI: 10.1124/dmd.119.089235
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Tables

Table 1. Absolute quantifications of housekeeping proteins in HLM and HLS9

		HLM (pmol/mg protein)			HLS9 (pmol/mg protein)		
	Genes	**Mean**	**SD**	**CV (%)**	**Mean**	**SD**	**CV (%)**
Conventional Housekeeping Proteins	GAPDH	187.56	47.20	25.17	217.26	38.01	17.50
	TUBB	10.13	5.54	54.68	4.31	2.52	58.44
	ACTB	3.32	2.41	72.52	2.99	1.48	49.50
Most stable proteins in HLM	PPIA	63.97	11.21	17.53	168.24	35.24	20.94
	PRDX4	66.37	12.65	19.06	59.92	11.15	18.61
	CAND1	3.51	0.68	19.41	2.49	0.68	27.41
	APEH	9.98	1.98	19.89	5.25	0.92	17.49
	PAICS	13.14	2.73	20.76	8.01	1.65	20.57
Most stable proteins in HLS9	TPI1	35.91	8.55	23.80	138.19	18.41	13.32
	PRDX1	150.31	40.63	27.03	179.04	25.72	14.37
	PGAM1	10.94	2.63	23.99	37.17	6.07	16.33
	PAK1IP1	0.60	0.13	21.76	0.68	0.11	16.72
	APRT	6.46	1.57	24.29	19.40	3.25	16.77

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Table 2. Absolute quantifications of cytosol and organelle membrane marker proteins in HLM and HLS9

		HLM (pmol/mg protein)			HLS9 (pmol/mg protein)				
		Mean	**SD**	**CV (%)**	**Mean**	**SD**	**CV (%)**	**HLM/HLS9**	**Localization**
organelles protein markers	**ATP1A1**	2.08	1.82	87.74	0.27	0.23	84.29	7.70	Plasma Membrane
	COX4I1	10.46	6.15	58.84	2.78	1.65	59.39	3.76	Mitochondria
	EPHX1	230.00	88.59	38.52	82.49	43.59	52.84	2.79	ER
	FTCD	194.81	67.15	34.47	87.08	30.88	35.46	2.24	Golgi apparatus
	PSMD1	8.03	2.00	23.82	2.85	0.89	27.00	2.82	Proteasome
	RPL7A	19.17	13.94	72.69	5.29	3.63	68.65	3.62	Ribosome
cytosolic protein markers	**TMSB4X**	5.16	2.61	50.57	39.66	20.98	52.90	0.13	Cytoskeleton
	AKR1A1	19.23	6.52	33.91	82.49	23.42	28.39	0.23	Cytosol

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Figures

Figure 1.

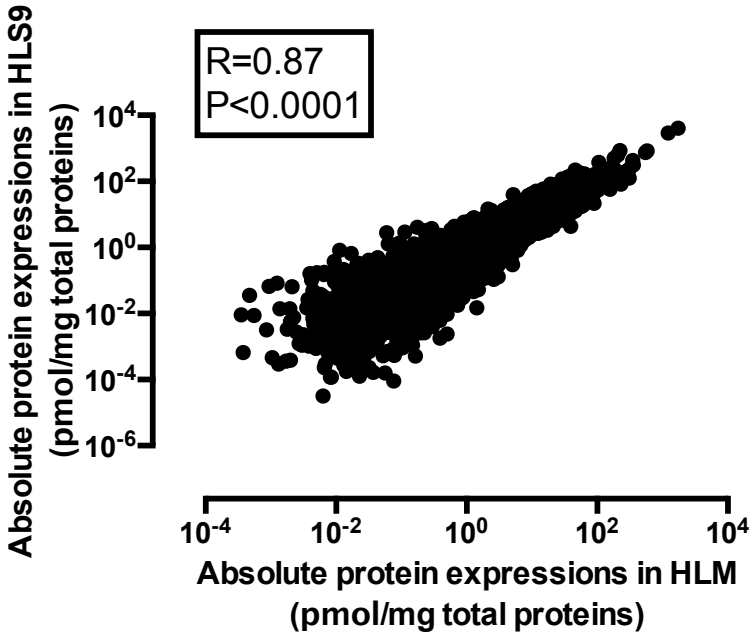

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Figure 2.

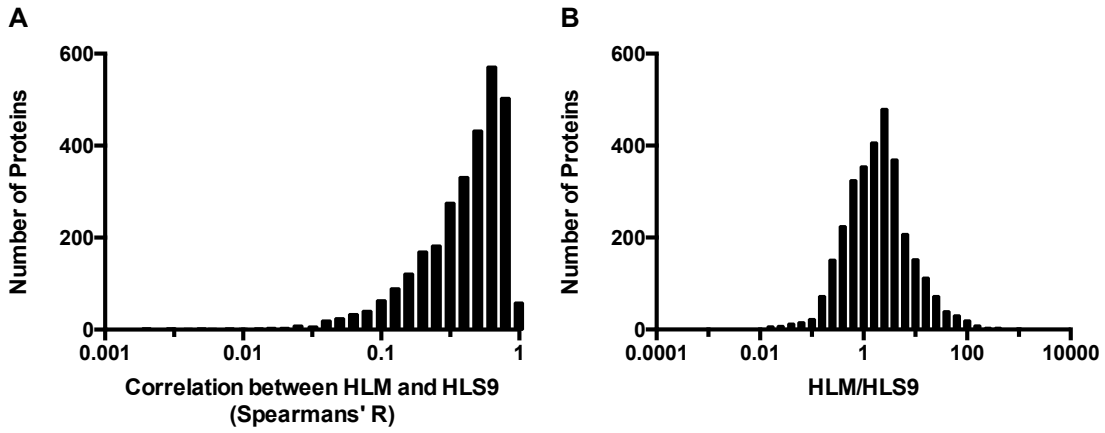

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Figure 3.

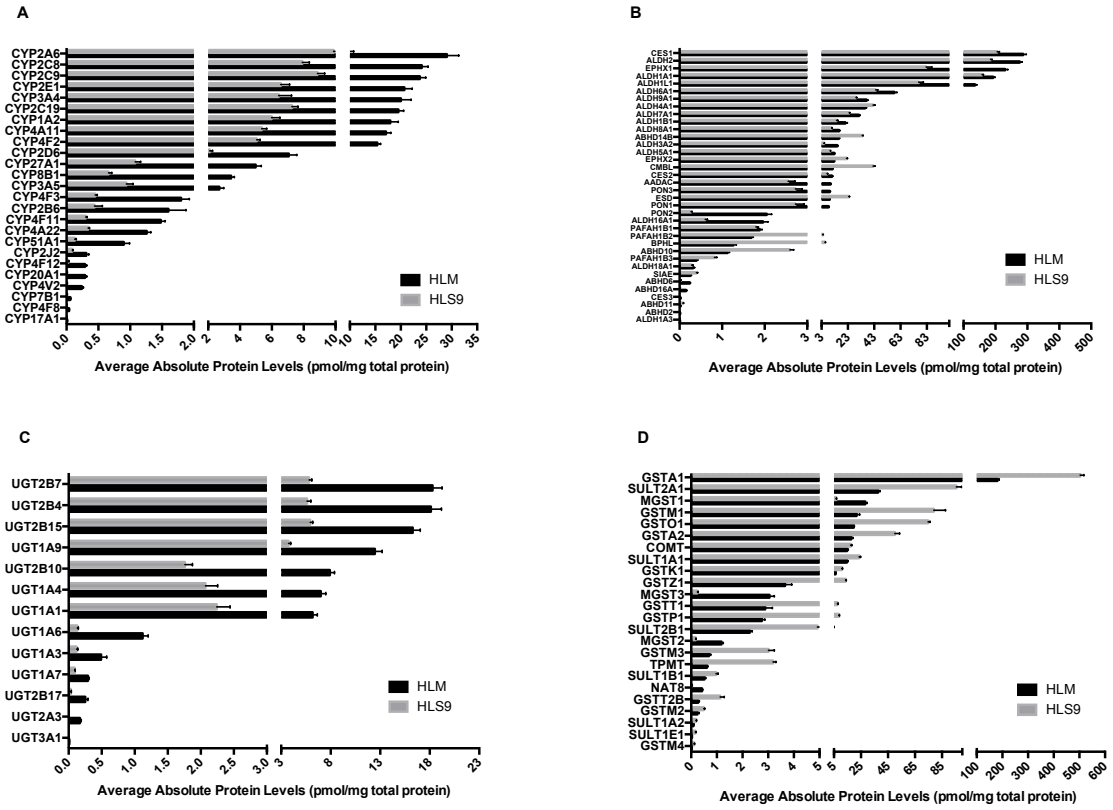

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Figure 4.

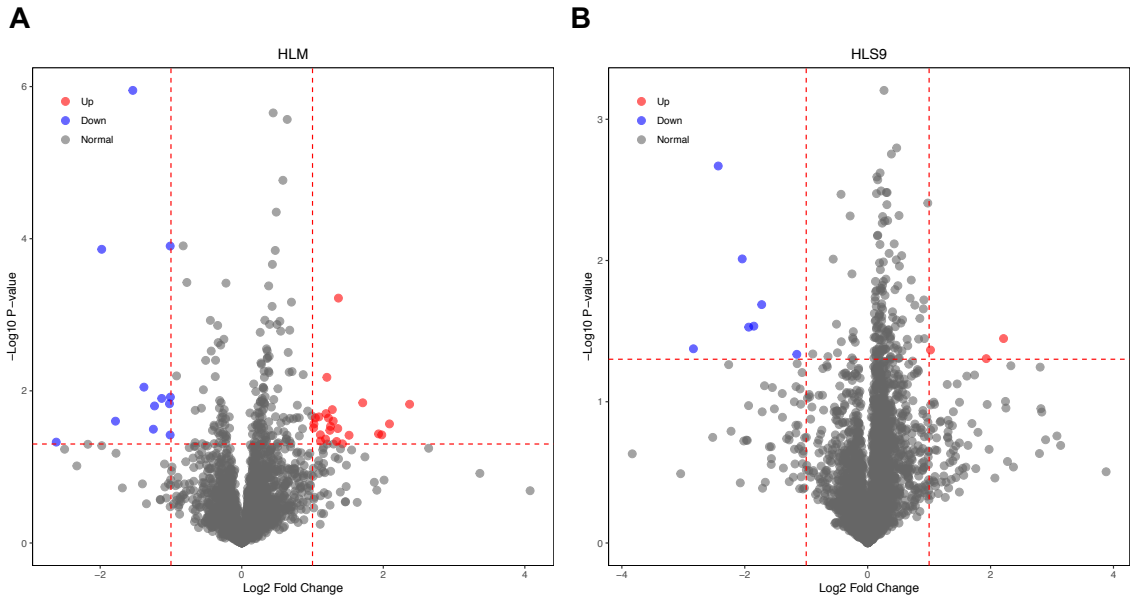

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Figure 5.