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Cell-type-resolved proteomic analysis of the human liver

Magnus Ölander[1] | Jacek R. Wiśniewski[2] | Per Artursson[3]

[1]Department of Pharmacy, Uppsala University, Uppsala, Sweden

[2]Biochemical Proteomics Group, Department of Proteomics and Signal Transduction, Max Planck Institute of Biochemistry, Martinsried, Germany

[3]Department of Pharmacy and Science for Life Laboratory, Uppsala University, Uppsala, Sweden

Correspondence
Per Artursson, Department of Pharmacy, Uppsala University, Box 580, SE-75123 Uppsala, Sweden.
Email: per.artursson@farmaci.uu.se

Abstract

Background & Aims: The human liver functions through a complex interplay between parenchymal and non-parenchymal cells. Mass spectrometry-based proteomic analysis of intact tissue has provided an in-depth view of the human liver proteome. However, the predominance of parenchymal cells (hepatocytes) means that the total tissue proteome mainly reflects hepatocyte expression. Here we therefore set out to analyse the proteomes of the major parenchymal and non-parenchymal cell types in the human liver.

Methods: We applied quantitative label-free proteomic analysis on the major cell types of the human liver: hepatocytes, liver endothelial cells, Kupffer cells and hepatic stellate cells.

Results: We identified 9791 proteins, revealing distinct protein expression profiles across cell types, whose in vivo relevance was shown by the presence of cell-type-specific proteins. Analysis of proteins related to the immune system indicated that mechanisms of immune-mediated liver injury include the involvement of several cell types. Furthermore, in-depth investigation of proteins related to the absorption, distribution, metabolism, excretion and toxicity (ADMET) of xenobiotics showed that ADMET-related tasks are not exclusively confined to hepatocytes, and that non-parenchymal cells may contribute to drug transport and metabolism.

Conclusions: Overall, the data we provide constitute a unique resource for exploring the proteomes of the major types of human liver cells, which will facilitate an improved understanding of the human liver in health and disease.

KEYWORDS
endothelial cells, hepatic stellate cells, hepatocytes, Kupffer cells, liver, proteomics

1 | INTRODUCTION

The liver is the largest internal organ in the human body and plays important roles in many vital metabolic and secretory processes. Most liver-specific functions, such as lipid metabolism, plasma protein synthesis and detoxification are mainly performed by parenchymal hepatocytes,[1] whose functionality depends on a complex interplay with different non-parenchymal cells (NPCs). Hepatocytes are arranged in cords between small capillaries known as sinusoids, lined by liver sinusoidal endothelial cells (LSEC). These are specialized liver endothelial cells (LECs) that facilitate direct contact between blood and hepatocytes through numerous fenestrations, and show high endocytic capacity.[2] The sinusoids also contain liver-resident macrophages called Kupffer cells (KC), which comprise as much as 80%-90% of the total macrophage population in the body.[3] The perisinusoidal space of Disse contains hepatic stellate cells (HSC) which store lipids and vitamin A, and help maintain extracellular matrix homoeostasis.[4] Together, these four cell types constitute lobules, the structural units of the liver microarchitecture. Hepatocytes represent 60% of the total liver cell population, and around 80% of liver volume, while most of the remaining population consists of LSEC, KC and HSC (and 6.5% of the liver volume, the remainder being vasculature and cholangiocyte-lined bile ducts).[5]

Advances in transcriptomics and proteomics have enabled system-wide investigations of gene and protein expression in human cells and tissues, including the liver.[6] Recently, laser-capture microdissection has also allowed the study of regional differences in gene expression throughout the liver microarchitecture.[7] However, since parenchymal cells constitute such a large proportion of the liver, whole-tissue studies mainly reflect hepatocyte expression. In contrast, single-cell RNA sequencing (RNA-seq) studies of dissociated liver cells have recently revealed several cellular subpopulations,[8-10] highlighting the importance of analysing multiple cell types to discern the intricacies of liver function.

Despite the insights gained from transcriptomics, a system-wide representation of the liver is not complete without proteomic information, as RNA levels only partly explain protein abundances.[11] Following this line of reasoning, the proteomes of all major cell types in murine liver have been analysed.[12,13] However, many liver functions show marked interspecies variability, and the applicability of rodent models for understanding human biology is increasingly coming into question.[14] This is problematic in studies of the absorption, distribution, metabolism, excretion and toxicity (ADMET) of drugs, where species differences in protein expression contribute to the poor predictive value of animal models.[15,16] Hence, proteomic analysis of the major cell types in human liver tissue would not only contribute to an improved general understanding of human liver function, but also be of use in drug discovery and development.

Here we therefore performed quantitative label-free proteomic analysis of human hepatocytes, LEC, KC and HSC. A unique feature of our study was that all four cell types originated from the same human donors, thus reducing the impact of variability arising from differences in patient background. Our analysis recapitulated functional differences between cell types, meaning that the data can

Key points

- Mass spectrometry-based proteomics was used to analyse the levels of almost 10 000 proteins in the four major cell types of the human liver: hepatocytes, liver endothelial cells, Kupffer cells and hepatic stellate cells.
- The data can be used as a resource to better understand human liver cell function.

be used to reliably probe various aspects of liver function, such as ADMET-related processes. Our data set thus constitutes a unique, quantitative resource for exploring global and cell-type-specific expression patterns in the proteomes of human liver cells.

2 | MATERIALS AND METHODS

2.1 | Human liver cells

Matched samples of cryopreserved hepatocytes, LEC, KC and HSC from three human donors with normal liver histology (Figure S1) were generously provided by Samsara Sciences (see Figure S1 for additional donor information). We use the term 'liver endothelial cells', rather than LSEC, since the provider cannot guarantee the absence of vascular endothelial cells. However, it should be noted that LSEC comprise the vast majority of endothelial cells in the liver,[17] and the isolation method used here has been shown to yield highly pure LSEC.[18] Human donor livers for cell isolation were collected using the same methods as for livers harvested for transplantation. All donor livers were obtained with informed consent of the donor or the donor family and in accordance with all US federal and state laws regarding organ donation. All donor information was redacted prior to arrival at Samsara Sciences. The livers were first flushed in situ through the aorta with 5-10 L of cold organ preservation solution (such as Belzer UW solution) at the time of harvest, and then removed from the body and flushed with an additional 2-5 L of cold organ preservation solution. The flushed livers were placed in sterile bags containing cold organ preservation solution and packed in two additional sterile bags. Finally, they were packed on ice in a shipping container and shipped via a courier service. The livers arrived at Samsara Sciences with <24 hours of cold ischaemia time.

The liver tissue was digested with a two-step collagenase perfusion protocol, as previously described.[19] Another 3-6 L of perfusion buffers were flushed through the livers during perfusion and digestion. Hepatocytes were isolated after digestion using low-speed centrifugation and purification through a Percoll gradient, followed by resuspension in a cryopreservation solution and freezing with a programmable controlled-rate freezer.[19] The supernatant from the first centrifugation step during hepatocyte isolation was used for the isolation of NPC types. LEC were isolated by centrifugation through an Optiprep gradient followed by elutriation.[18] LEC were collected as a band at the

interface of the Optiprep gradient and the cushion of Hank's balanced salt solution (HBSS) above. The collected cells were washed with HBSS and centrifuged to remove remaining Optiprep, followed by processing through the elutriator. Purified LEC were cultured in collagen I coated flasks using EGM-2 medium (Lonza), and allowed to grow to confluence (approximately 7-14 days) with medium exchanges every third day. Once confluent, the cells were removed from the culture flasks using a combination of trypsin (0.25%) and EDTA (2.21 mmol/L) at 37°C for 3-6 minutes. The LEC were then centrifuged, resuspended in EGM-2 medium, counted and centrifuged again. LEC were resuspended in 90% FBS +10% DMSO and cryopreserved as passage 0 cells using Corning CoolCell freezing containers. Passage 0 LEC were quickly thawed at 37°C, mixed with five volumes of EGM-2 medium, and centrifuged. The cell pellet was resuspended in EGM-2 medium and LEC were cultured as passage 1 cells. The cells were allowed to grow to >85% confluence, then passaged and grown again to >85% confluence. These LEC were removed using trypsin/EDTA as described above, washed, counted and cryopreserved as passage 2 cells at a concentration of 1.2×10^6 cells/mL. KC were isolated using CD11b magnetic beads and cryopreserved immediately after isolation using Corning Coolcell freezing containers. HSC were isolated by centrifugation through a Nycodenz gradient.[20] Isolated HSC were cultured and grown to confluence, then cryopreserved as passage 0 cells using Corning CoolCell freezing containers. Importantly, the methods used for cell isolation by the provider have previously been shown to yield cells of high purity.[18-20]

2.2 | Initial characterization of isolated cells

Characteristics and purity of the isolated cells were assessed by Samsara Sciences (see Data S1 for cell characterization). Hepatocyte fractions contained very small percentages of mixed NPCs after Percoll purification, as determined by microscopic analysis of cultured hepatocytes. LEC were characterized by flow cytometry using the markers CD-31, CD-299, CD-45, vWF, CD-146, and LYVE-1. KC were stimulated by LPS and characterized via expression of the cytokines IFN-γ, IL-1B, TNF, IL-2, IL-4, IL-6, IL-8, IL-10 and IL-12p70 using the Meso Scale Discovery (MSD) instrument. KC purity was determined by fluorescent staining for CD-11b and CD-68 (data not shown). HSC characterization included fluorescent staining for the stellate markers desmin, GFAP and α-SMA, and the non-stellate markers TE-7 (fibroblasts) and CD-31 (endothelial cells).

2.3 | Sample preparation

Thawed cell samples were homogenized on ice with 1% SDS in 0.1 mol/L Tris-HCl, pH 8.0, containing 0.05 mol/L DTT, and lysed as previously described.[21] Total protein amounts were determined using a tryptophan fluorescence assay in microtitre plate format.[22] Sample aliquots containing 15 μg protein were processed by MED-FASP using consecutive protein digestion with LysC, trypsin and chymotrypsin, as previously described.[23]

2.4 | LC-MS/MS analysis

Analysis of peptide mixtures was performed using a Q Exactive HF-X mass spectrometer (Thermo Fisher Scientific). Aliquots containing 1 μg of total peptide were chromatographed on a 50 cm column with 75 μm inner diameter packed with C18 material. Peptide separation was carried out at 300 nL/min for 95 min using 5%-30% of an acetonitrile gradient. The temperature of the column oven was 55°C. To minimize carryover between samples of different origin, two 'empty runs' were applied after each cell type. The mass spectrometer was operated in data-dependent mode with survey scans acquired at a resolution of 60 000. Up to the top 15 most abundant isotope patterns with charge ≥+2 from the survey scan (300-1650 m/z) were selected with an isolation window of 1.4 m/z and fragmented by HCD with normalized collision energies of 25. The maximum ion injection times for the survey scan and the MS/MS scans were 20 and 28 ms respectively. The ion target values for MS1 and MS2 scan modes were set to 3×10^6 and 1×10^5 respectively. The dynamic exclusion was 30 seconds. The mass spectrometry proteomics data has been deposited to the ProteomeXchange Consortium via the PRIDE[24] partner repository with the data set identifier PXD012615.

2.5 | MS data analysis

Mass spectrometric data was analysed using the MaxQuant software.[25] A maximum of two missed cleavages was allowed. The maximum false peptide and protein discovery rate were specified as 0.01. To ensure that the carryover controls did not include significant amounts of residual peptides, every second 'empty run' was analysed as well. Protein concentrations were calculated with the Total Protein Approach,[26] using raw intensity values from the MaxQuant output.

2.6 | Statistical analysis

Pathway analysis was performed with the Reactome database, version 67.[27] Transcription factor (TF) predictions were performed using the FunRich software, version 3.1.3.[28] Principal component analysis (PCA) was performed with SIMCA, version 15.0.0.4783. Statistical significance tests were carried out with Excel.

3 | RESULTS

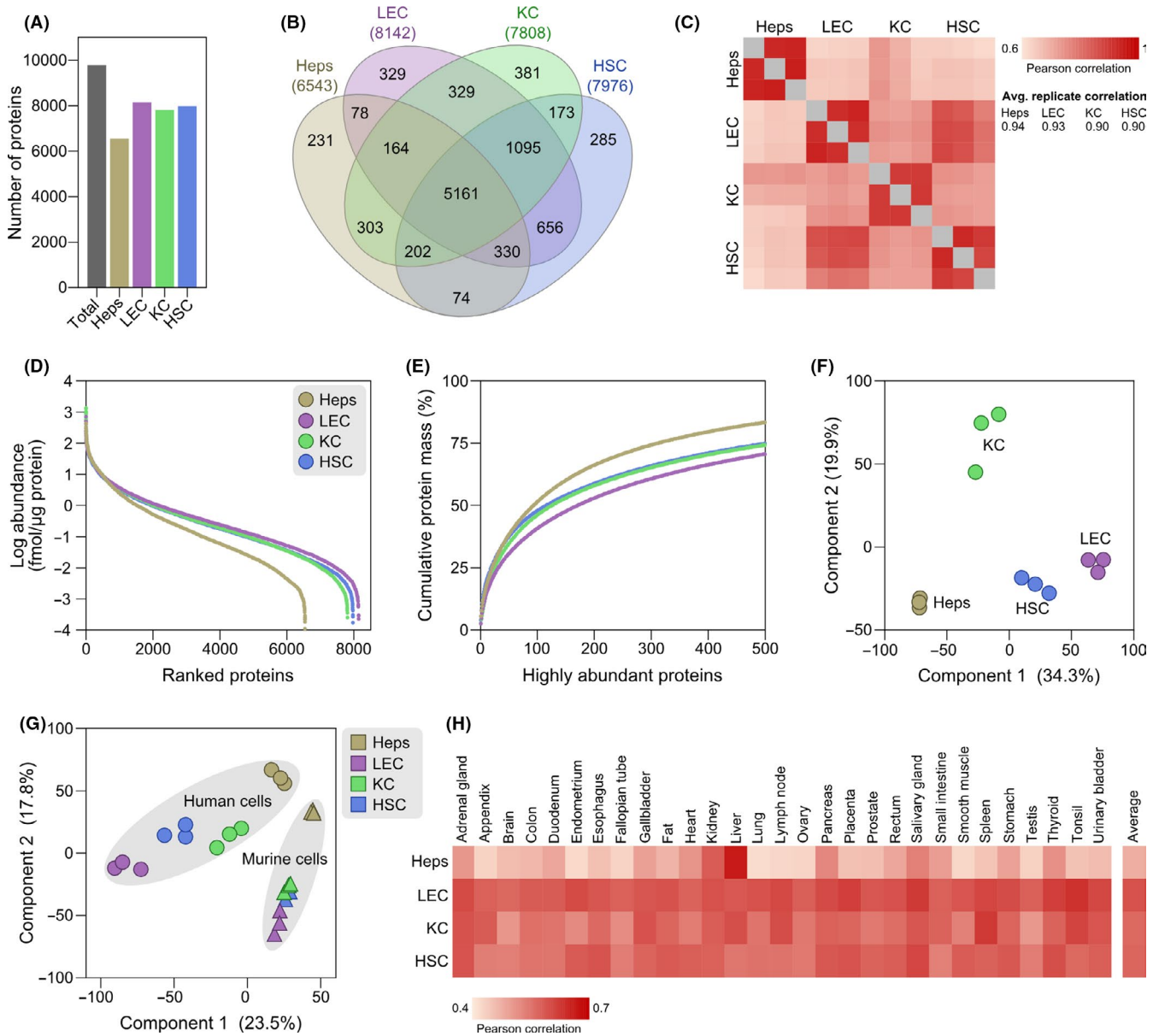### 3.1 | Proteomic analysis of the major cell types in human liver

To analyse the proteomes of the major cell types in human liver, we obtained matched samples of isolated hepatocytes, LEC, KC and HSC from three donors (see Data S1 for cell characterization). All donor livers were histologically normal (Figure S1), with NAFLD activity scores[29] of 0. High purity of the different cell populations had

previously been verified by the provider (>95% for hepatocytes and HSC; >90% for LEC and KC). In total, our label-free quantitative proteomic analysis of biological triplicates for each cell type resulted in the identification of 9791 proteins (Figure 1A; Table S1). Of these, 6543 were identified in hepatocytes, whereas approximately 8000 proteins were found in the different NPCs. Around 53% of proteins (5161) were identified in all cell types, indicating shared basal cellular processes (Figure 1B). Proteins essential for the survival of human cell lines[30] showed an even higher overlap; 80.5% of the 1473 essential proteins we detected were common to all cell types, and 89.5%

were found in at least three cell types (Figure S2). The reliability of our data was indicated by strong correlations among biological replicates of the individual cell types (average Pearson's r of .90-.94; Figure 1C). Cellular proteomes covered similar dynamic ranges of around seven orders of magnitude (Figure 1D). The 500 most highly abundant proteins accounted for 83% of the total protein mass in hepatocytes, but only 71%-75% in NPCs, supporting the functional specialization of the hepatocyte proteome (Figure 1E). Furthermore, PCA of the proteomes showed that the cell types all had distinctly different proteomes (Figure 1F). The hepatocyte samples from the

FIGURE 1 Proteomic analysis of the four major cell types in the human liver. A, Number of proteins identified in the different cell types. B, Overlap in protein identifications between cell types. C, Correlations of global protein expression among biological replicates. All cell types from three donors were analysed. D, Ranked protein concentrations in the four cell types, showing the dynamic range of the analysis. E, Cumulative protein mass for the 500 most highly abundant proteins in each cell type. F, Principal component analysis of the cellular proteomes. The numbers in parentheses show the amount of variability explained by each component. G, Species differences between human and murine liver cells, visualized by principal component analysis, using our human data and a previously published murine data set.[13] H, Liver cell proteomes compared with the proteomes of 29 human tissues.[6]
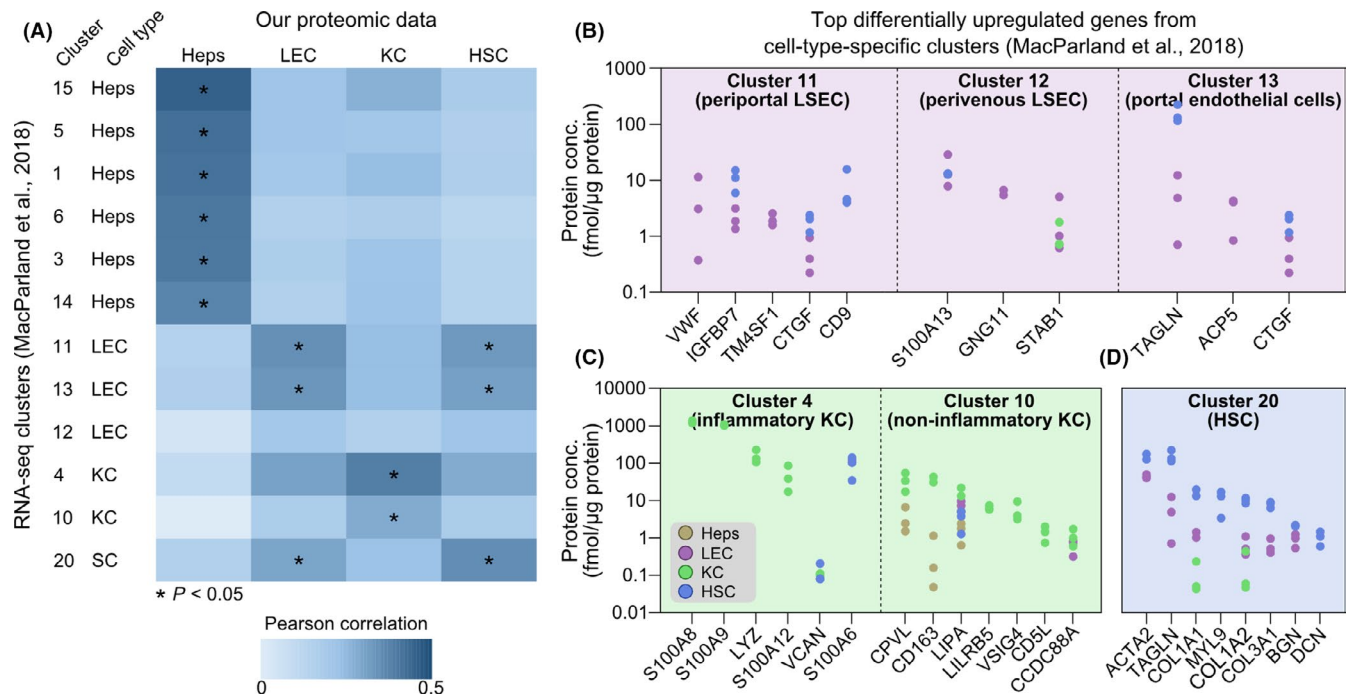
FIGURE 2 Comparison with single-cell RNA sequencing data of human liver cells. A, Protein concentrations in our data set compared with corresponding RNA levels, using the available data from an RNA-seq study of dissociated human liver cells.[9] Cluster numbers denote cell type designations in the RNA-seq study. B-D, Protein concentrations of the top differentially upregulated genes in (B) LEC, (C) KC and (D) HSC in the RNA-seq study

three donors were clustered apart from the corresponding NPCs, in line with the results described above.

To further characterize proteomic differences between cell types, we compared our data with a previous proteomic analysis of the same cell types in murine liver.[13] The comparison was performed using values for the fraction of total protein represented by each individual protein (such values were calculated by TPA here, and were also available for the murine data set). Proteins that were found in both data sets were included in the comparison. PCA revealed clustering by species (Figure 1G). Interestingly, human and murine hepatocytes were fairly similar, whereas NPCs tended towards larger species differences. This indicates partly divergent roles of these cell types in the two species and emphasizes the importance of studying human cells to understand human liver biology. We also compared our data with a recent study of the proteomes of 29 human tissues.[6] Hepatocytes were generally less similar than NPCs to other tissue proteomes ($r = 0.48$, on average, compared to 0.57-0.60 for NPCs), further accentuating the high level of functional specialization of hepatocytes (Figure 1H). For convenience, our proteomic data of human hepatocytes, LEC, KC and HSC are provided in an easily accessible format in Table S1.

3.2 | Comparison with single-cell RNA sequencing data

Recently, sensitive liver dissociation techniques were used together with RNA-seq to create a transcriptomic map of the human liver.[9]

The study identified 20 discrete subpopulations (clusters) of different liver cells. We compared RNA levels with protein concentrations in our data set, using the available data on differentially expressed genes in each cluster. In general, the highest similarities were observed between matching cell types, with RNA/protein correlations of 0.20-0.45 (Pearson's r; Figure 2A). Hepatocytes showed stronger correlations than NPCs, in line with previous findings in murine liver cells.[13] LEC and HSC showed significant correlations with each other as well, indicating shared expression patterns. The embryonic origin of HSC has been controversial, but it has previously been suggested that LSEC and HSC share a common origin.[12]

Furthermore, we selected the top 10 differentially upregulated genes (compared to global expression in all cells) from the NPC-specific RNA-seq clusters[9] and investigated the corresponding protein concentrations of genes that were also found in our analysis. To ensure reliable quantification, we only considered proteins that were identified by at least three unique peptides in all samples of a certain cell type. Among the 32 proteins that thus remained, the majority (25) showed the highest expression in the expected cell types (Figure 2B-D). For example, the endothelial marker von Willebrand factor (VWF) was unique to LEC, the macrophage marker CD163 was highest in KC, and the stellate cell marker alpha-smooth muscle actin (ACTA2) was highest in HSC. Discrepancies were mostly accounted for by the proteomic similarities between LEC and HSC, where HSC showed similar or higher levels of some proteins that were predominant in LEC on the RNA level. Overall, however, these results indicate that cell-type-defining genes at the RNA level show similar patterns at the protein level.

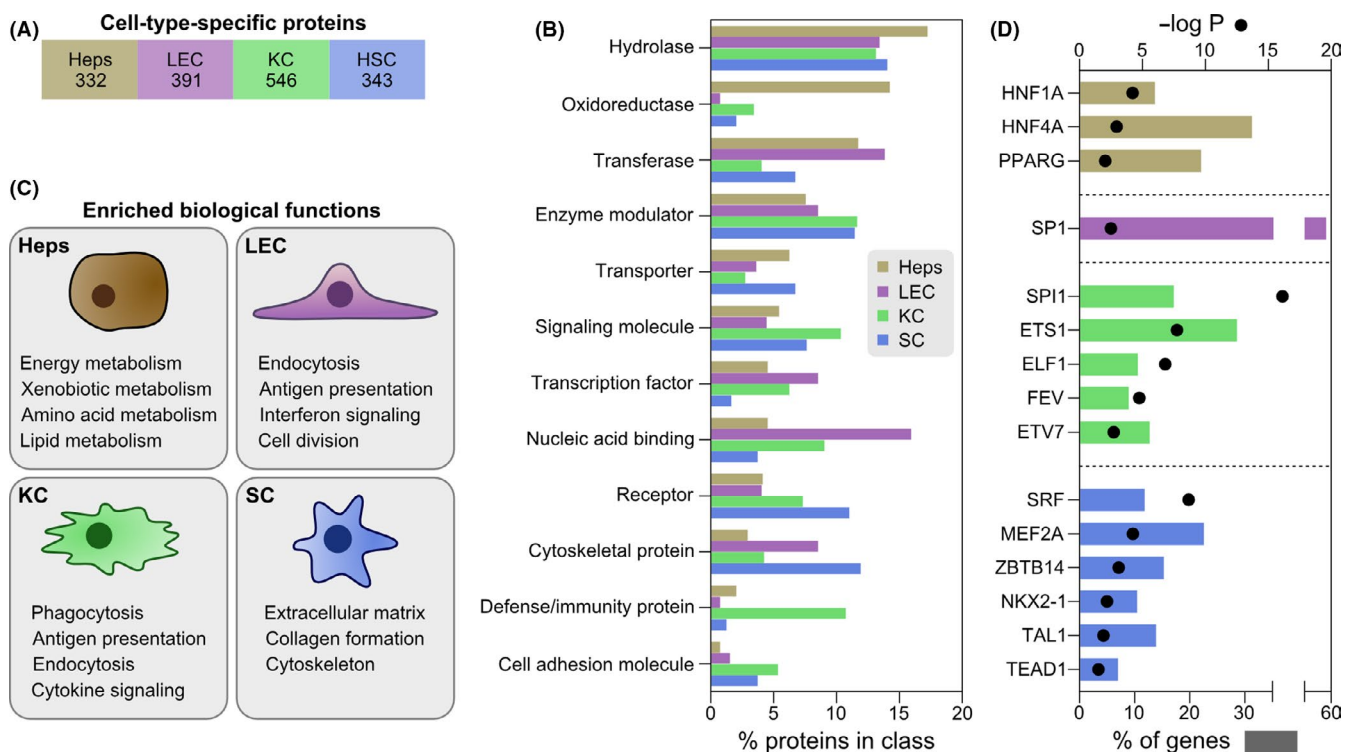### 3.3 | Cell-type-specific proteins in human liver

We then studied the characteristics of cell-type-specific proteins. We included proteins that were unique to one cell type or showed at least 50-fold higher levels in one cell type compared to the others. This resulted in around 300-500 cell-type-specific proteins for each cell type (Figure 3A; Table S2). We used the PANTHER classification system[31] to categorize these by protein class. For a clearer picture, we only considered classes that comprised at least 5% of cell-type-specific proteins in at least one cell type (Figure 3B). Hepatocyte-specific proteins were largely classified as metabolic enzymes, for example, hydrolases, oxidoreductases and transferases. On the other hand, many NPC-specific proteins were of a more regulatory nature, with a high representation in classes such as enzyme modulators, signalling molecules and TFs. Moreover, we performed pathway analysis of the cell-type-specific proteins, to investigate which biological functions they represented. As expected, many hepatocyte-specific proteins were involved in the metabolism of xenobiotics, amino acids and lipids (Figure 3C; Table S3). Proteins specific to LEC and KC were largely associated with immune system functions, including antigen presentation and endocytosis. Phagocytosis was unique to KC, in line with their macrophage phenotype. LEC also showed upregulation of many proteins involved in cell division, possibly related to the proliferative response associated with liver regeneration after hepatectomy.[32] We note that this somewhat obscures the LEC data, but our comparative analyses show that the

data still provides biologically relevant information on the composition of the LEC proteome. Finally, HSC-specific proteins were involved in the organization of the extracellular matrix and the cytoskeleton. We anticipate that the cell-type-specific proteins found here will be useful to identify new markers for cell sorting and characterization.

Next, we performed predictions of which TFs were involved in regulating the expression of cell-type-specific proteins. Significant TFs were found for all cell types (Figure 3D), of which certain interesting cases are worth highlighting. For hepatocytes, HNF1A and HNF4A were involved, which regulate many liver-specific functions,[33] as well as PPARG, a regulator of hepatic lipid metabolism.[34] KC-specific proteins were regulated by several TFs of importance for innate and adaptive immunity, including SPI1, ETS1 and ELF1.[35,36] HSC-specific proteins were, among others, regulated by SRF, which plays a critical role in the development of mesoderm-derived cells,[37] and MEF2A, which mediates HSC activation and fibrogenesis.[38] Together, the TF and pathway analyses showed that cell-type-specific proteins generally represented functions performed by different human liver cells in vivo, further demonstrating the validity and usefulness of our data.

3.4 | Proteins involved in the immune system

Drug-induced liver injury (DILI) is a major problem for public health and in drug development.[39] The most problematic form is referred

FIGURE 3 Analysis of cell-type-specific protein expression. A, Number of cell-type-specific proteins identified in the different cell types. Proteins were considered cell-type-specific if unique to one cell type or at least 50-fold higher in one cell type than in the other three. B, Protein classes of cell-type-specific proteins, categorized with the PANTHER classification system. C, Enriched biological functions in the different cell types. D, Transcription factors predicted to participate in regulating the expression of cell-type-specific proteins. Statistical significance (-log P) is plotted on the top axis, and the percentage of cell-type-specific proteins regulated by different transcription factors is plotted on the bottom axis

FIGURE 4 Expression of proteins involved in the immune system. A-E, Protein concentrations of (A) antigen presentation proteins, (B) cytokine receptors, (C) the complement system, (D) pattern recognition receptors and (E) alarmins (endogenous DAMPs). ND, not reliably detected (see main text)
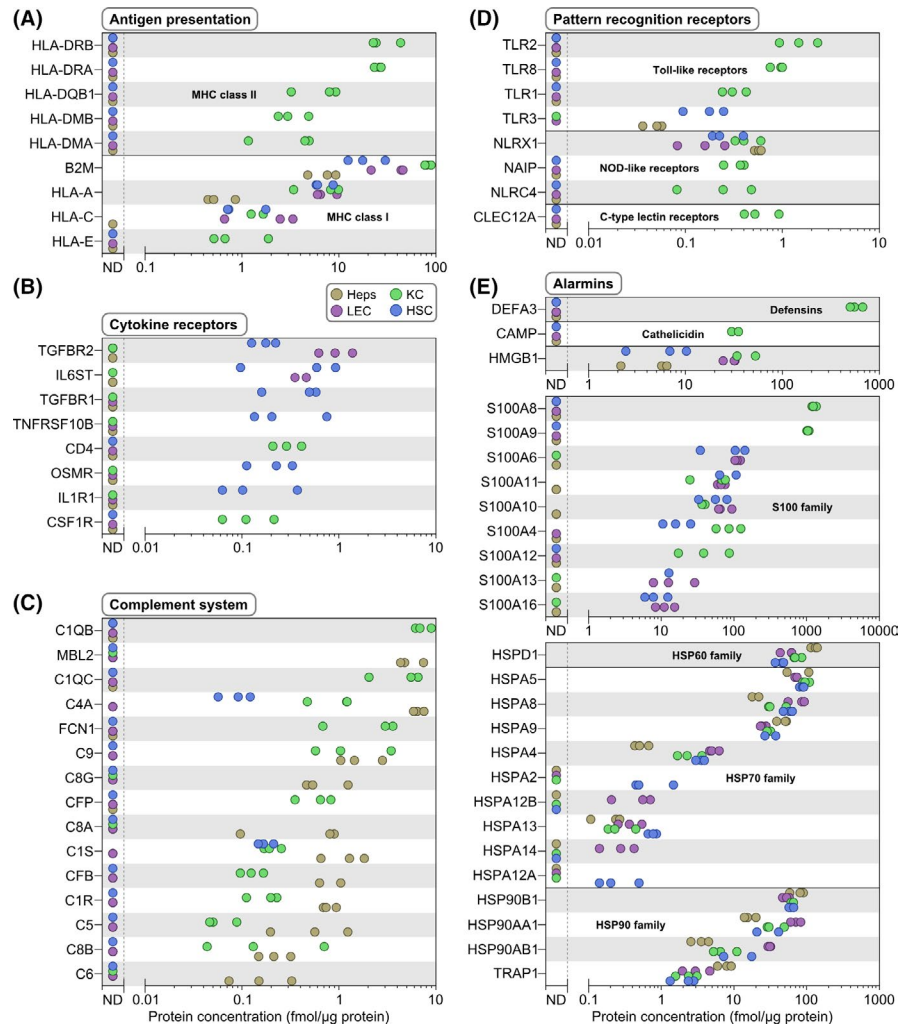
to as idiosyncratic DILI, where hepatotoxicity occurs with prolonged latency in a highly unpredictable manner. Idiosyncratic DILI is often connected to an immune response triggered by the offending drug, with sequential involvement of innate and adaptive immune responses that eventually cause hepatocyte death.[39] To investigate the expression patterns of proteins potentially involved in idiosyncratic DILI, we investigated the expression of selected categories of proteins with important immune functions, including antigen presentation proteins, cytokine receptors, the complement system, pattern recognition receptors (PRRs) and alarmins. As in the section 'Comparison with single-cell RNA sequencing data', we only considered proteins that were detected by at least three unique peptides in all samples of a cell type.

Firstly, as expected, expression of the antigen-presenting major histocompatibility complex (MHC) class I proteins was distributed across cell types, whereas MHC class II proteins were only found in KC (Figure 4A). Secondly, the eight cytokine receptors we detected were only expressed in NPCs, notably in HSC (Figure 4B). For instance, HSC expressed TGF-β receptors (TGFBR1 and TGFBR2), the oncostatin M receptor (OSMR) and an interleukin-1 receptor (IL1R1), whose ligands all have fibrogenic effects in these cells.[40]

Thirdly, proteins from the complement system were mainly detected in hepatocytes and KC (Figure 4C). Fourthly, PRRs were mainly expressed by KC (Figure 4D), including exclusive expression of three Toll-like receptors (TLR2, TLR8 and TLR1), two NOD-like receptors (NLRs; NAIP and NLRC4) and a C-type lectin receptor (CLEC12A). Exceptions were TLR3, which was found in hepatocytes and HSC, and NLRX1, which was expressed in all cell types. Fifthly, we found that KC showed exclusive expression of several prominent alarmins (Figure 4E), which are endogenous damage-associated molecular patterns (DAMPs),[41] including a defensin (DEFA3), cathelicidin (CAMP) and the most important inflammatory members of the S100 family (S100A8, S100A9 and S100A12). Heat shock proteins (HSPs), which can also act as alarmins, were more similar across cell types, likely owing to their essential roles in basic cellular function (Figure 4E). In summary, we found that KC were the most prominent cells in terms of immune-related protein expression, in line with their roles as liver-resident macrophages. However, some immune-related proteins were predominantly expressed in other cell types, which suggests that our proteomic data can be helpful in providing informed starting points for in-depth studies of idiosyncratic DILI.

3.5 | Proteins involved in drug disposition

The liver is the most important organ for detoxification of xenobiotics in the human body. Hence, we studied the expression of proteins involved in the ADMET of drugs in the different cell types, using a previously compiled set of 683 ADMET-related genes.[42] Again, we only included proteins that were detected by at least three unique peptides in all samples of a cell type. About 283 of these genes were retained in our comparison (Table S4). To compare the NPCs with hepatocytes, the most well-known cell type in terms of ADMET, we ranked the proteins by their abundance in hepatocytes. An interesting pattern emerged, where around a third of the proteins showed higher expression in hepatocytes, whereas another third were generally higher in NPCs (Figure 5A). Hence, we divided the ranked list into three equal quantiles of 94 proteins each and classified the proteins with PANTHER. Almost half of the proteins (47%) from the first quantile (with predominant expression in hepatocytes) were oxidoreductases (Figure 5B), compared to only 5% in the last quantile (with predominant expression in NPCs; Figure 5C). This broad group of enzymes contains many important detoxifying enzymes, such as the cytochrome P450s (CYPs), aldehyde dehydrogenases and alcohol dehydrogenases. Proteins in the last quantile were more evenly distributed across classes. As in the analysis of cell-type-specific expression, many of these were regulatory proteins, prominently TFs and nucleic acid binding proteins (Figure 5C).

We next specifically examined solute carrier (SLC) and ATP-binding cassette (ABC) transporters of importance for uptake and efflux of drug molecules. For this purpose, we used a list of 36 clinically relevant transporters expressed by ADMET-relevant tissues, as defined by the International Transporter Consortium.[43] We identified 16 of these transporters here, using the strict criterion described above, out of which 12 showed predominant expression in hepatocytes (Figure 5D,E). The most highly expressed clinically relevant SLC transporters in hepatocytes were SLCO1B1, SLC22A1, SLCO1B3 and SLCO2B1, in agreement with previous studies.[44-46] Unexpectedly, the solute carrier organic anion (SLCO) transporters, usually considered hepatocyte-specific in the liver, were also detected in KC. This prompted an investigation into possible hepatocyte contamination of the KC samples, as their purity was around 90%. To this end, we analysed all cell types for their expression of the 93 marker proteins included in the liver-specific gene expression panel (LiGEP), which is dominated by hepatocyte-specific proteins.[47] Indeed, 70 of the 84 markers we detected were also found in the KC samples, albeit at lower levels (Figure S3). Furthermore, the markers showed similar expression patterns in hepatocytes and
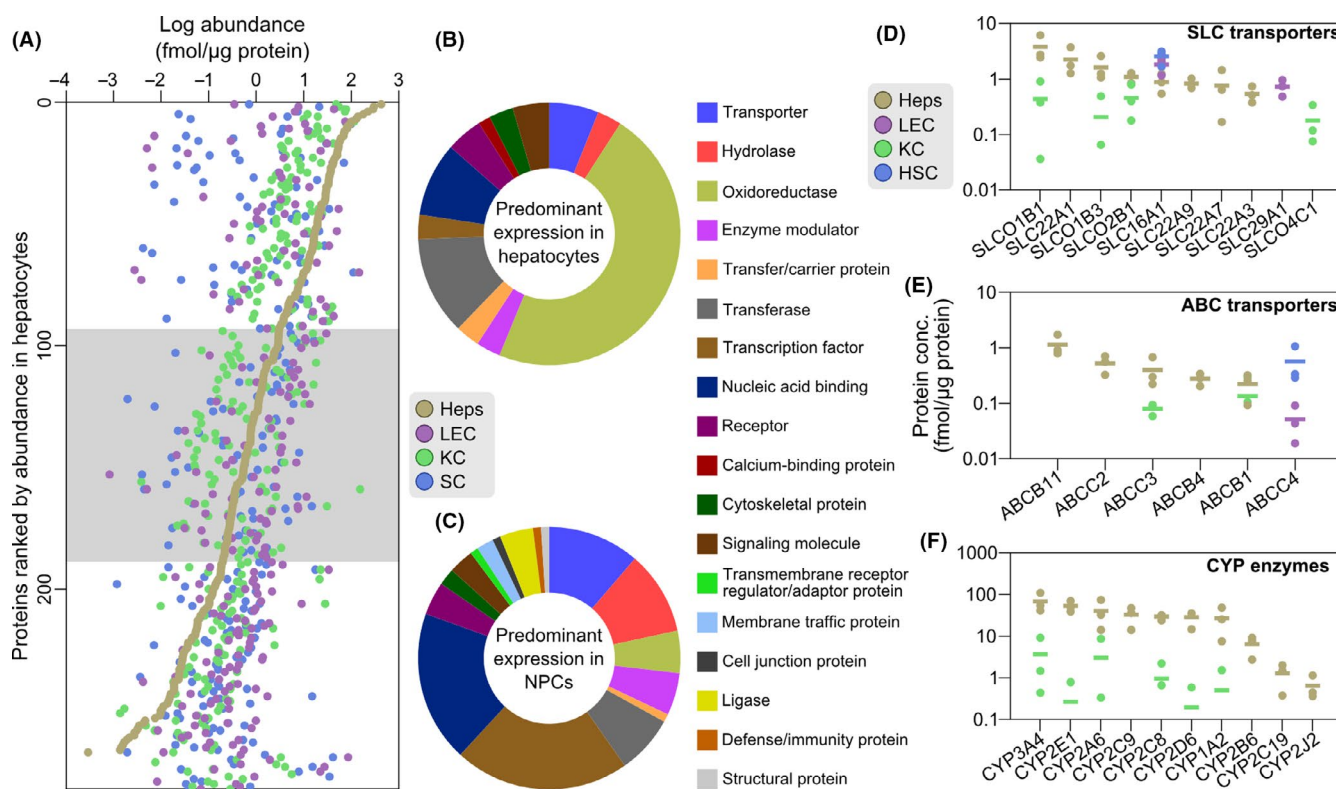
FIGURE 5 Expression of proteins involved in drug disposition. A, ADMET-related proteins in hepatocytes, LEC, KC and HSC, ranked by the abundance in hepatocytes. The plot is divided in three quantiles of 94 proteins each. The middle quantile is shown in grey. B-C, PANTHER protein classification of ADMET-related proteins from (B) the first quantile, with predominant expression in hepatocytes, and (C) the last quantile, with predominant expression in NPCs. D-F, Protein concentrations of (D) SLC transporters, (E) ABC transporters and (F) CYP enzymes in the four different cell types. Expression in KC was corrected for hepatocyte contamination, as described in the text and detailed in Table S5

KC, with Spearman correlation coefficients of 0.92, 0.87 and 0.81 in the three donors, respectively, which was not observed for LEC and HSC, indicating hepatocyte contamination of the KC samples. We also analysed LiGEP panel expression in the data from two comparable studies of murine liver cell proteomes.[12,13] Interestingly, around 80% of the LiGEP proteins that were detected in these studies were found in all cell types, and at relatively similar (within fivefold, on average) levels to hepatocytes (Figure S4). This shows that possible contamination problems because of suboptimal purification or analysis are not limited to our data set. In fact, the different cell types showed higher degrees of separation in our data than in the murine studies (Figure 1G; Figures S3 and S4), likely as a result of the rigorous procedure we followed to keep the cells separated in the mass spectrometry analysis.

To account for hepatocyte contamination of the KC samples in our data set, we calculated correction factors based on the difference in LiGEP protein expression between the two cell types (Table S5). We then used these correction factors to correct KC transporter expression, resulting in the values presented in Figure 5D,E. Some SLCO expression remained even after the correction, however, suggesting that these transporters might be expressed by KC. This is not inconceivable, since at least SLCO2B1 and SLCO4C1 (SLCO4C1 was KC-specific here) are expressed by human blood macrophages.[48] Regarding the ABC transporters, hepatocytes showed the highest expression of all but one, ABCC4, which was detected in HSC and LEC in our analysis, with the highest levels in HSC (Figure 5E). Full expression profiles of SLC and ABC transporters in the different cell types are given in Figures S5 and S6.

Furthermore, we investigated the expression of the 10 most important CYP enzymes in hepatic drug metabolism, that is, CYP3A4, CYP2D6, CYP2C9, CYP1A2, CYP2B6, CYP2C19, CYP2C8, CYP2A6, CYP2E1 and CYP2J2.[49] As expected, hepatocytes showed the highest expression levels of all of these CYPs (Figure 5F). Curiously, however, six of the CYPs were also present in KC, even after correcting for hepatocyte contamination as described above, possibly indicating an active role of these cells in drug metabolism.

Some of our findings on ADMET-related proteins (eg SLCO2B1 and ABCC3 in KC) could be confirmed on the mRNA level using a publicly available human liver single-cell RNA-seq data set navigation tool,[9] while some (eg CYP enzymes in KC) were more ambiguous. However, it should be noted that mRNA and protein concentrations are often not strongly correlated.[11] Functional studies will be required to definitively verify the presence or absence of important ADMET-related proteins in NPCs. In summary, our proteomic analysis generally indicates that hepatocytes perform the majority of ADMET-related tasks, while NPCs have other functions.

4 | DISCUSSION

The proteome of the intact human liver has been well characterized.[6,44,46] However, these analyses mainly reflect parenchymal protein expression, as hepatocytes comprise the vast majority of liver tissue. Accordingly, comparative analysis showed that global protein abundances in liver and isolated hepatocytes were strongly correlated.[44] In this study, we therefore performed quantitative proteomic analysis of the major cell types in the human liver, that is, hepatocytes, LEC, KC and HSC, to offer insight into the nuances of liver function at the protein level. Our results reflect the expression patterns of known marker proteins and certain cell-type-specific liver functions. The comprehensive proteomic data we provide will thus be helpful for researchers interested in different aspects of human liver biology.

Similar analyses have previously been performed for the corresponding cell types in murine liver.[12,13] While these studies undoubtedly provide valuable insight into general aspects of mammalian liver function, our comparison with human liver cell proteomes shows that there are significant species differences and that proteomic analysis of human liver cells is required to understand human biology. This species discrepancy has previously been observed on the transcriptomic level, where different human tissues were found to be more similar to each other than to comparable murine tissues.[50] Comparative studies of specific aspects of liver biology, such as non-alcoholic fatty liver disease (NAFLD) progression[51] and CYP-mediated drug metabolism[16] further emphasize the uncertain translational value of mouse experiments. Nevertheless, an interesting aspect of one of the mouse studies is the inclusion of cholangiocytes.[12] Cholangiocytes line the bile ducts of the liver and are important for bile modification and the liver immune response.[52] These cells were not included here, as we focused on the cell types that constitute the liver lobule. However, proteomic analysis of human cholangiocytes would help provide an even more comprehensive view of the human liver proteome.

Interestingly, we noticed that there was a tendency towards larger proteomic differences among human liver cell types than among their murine counterparts (Figure 1G). In line with this, single-cell RNA-seq recently revealed the presence of 20 discrete cell populations in the human liver, including subpopulations of hepatocytes, LEC and KC.[9] Single-cell RNA-seq studies of murine liver cells did not identify as many subpopulations,[8,10] potentially indicating a higher level of complexity in human tissue. Indeed, such complexity has been observed on an organism-wide level, with higher degrees of cell and tissue specialization in humans.[53] Technical limitations currently preclude the application of single-cell proteomics in a similar manner to the transcriptomic studies mentioned above. Furthermore, it is not yet possible to isolate the 20 different subpopulations of human liver cells[9] in sufficient quantities for contemporary proteomic analysis, which would be required for the analysis of a fully cell-type-resolved liver proteome. However, our study constitutes the most comprehensive proteomic mapping of the major cell types in the human liver at this point.

One of the most important liver functions is the detoxification of xenobiotics. Thus, we specifically investigated proteins involved in ADMET-related processes. In general, the expression patterns indicated that hepatocytes perform most of the

uptake and metabolism of xenobiotics, while NPCs play more modulating roles, for example, through the expression of regulatory proteins. This is in line with observations from studies of hepatocytes in coculture with NPCs, where the regulatory effects of NPCs were demonstrated by prolonged maintenance of hepatocyte differentiation and functionality, for example, in terms of albumin secretion, CYP metabolism and the response to inflammatory and hepatotoxic stimuli.[54,55] Nevertheless, some clinically relevant transport proteins[43] were only detected in NPCs in our analysis. Specifically, SLC29A1 was only detected in LEC, SLCO4C1 was exclusive to KC and ABCC4 was only found in LEC and HSC. ABCC4 has previously been found at relatively high levels in human HSC.[56] Surprisingly, we also found that KC showed expression of several CYP enzymes of importance in drug metabolism, even after correcting for hepatocyte contamination. Macrophages in human blood, bone marrow and lung express several important CYPs,[57-59] so it is not inconceivable that these enzymes might also have a function in human KC. Functional studies of drug uptake and metabolism in the different human NPCs will shed more light on the functional relevance of our ADMET-related findings.

In conclusion, we used quantitative label-free mass spectrometry to analyse the proteomes of human hepatocytes, LEC, KC and HSC, thus providing a proteomic foundation for deepened understanding of human liver cell function. The cell-type-specific nature of our data will be beneficial for the design of functional studies, and will facilitate the interpretation of experimental results obtained using human liver cells. Full understanding of the effects of cellular perturbation and hepatic disease will require analysis of all subpopulations of liver cells. In the meantime, however, our data can serve as a benchmark for studying conditions that involve one or more of the four major cell types analysed here, thereby taking steps towards improved insight into human liver biology in health and disease.

CONFLICT OF INTEREST
The authors declare no conflict of interest.

ORCID
Magnus Ölander https://orcid.org/0000-0002-4502-8184
Per Artursson https://orcid.org/0000-0002-3708-7395

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.