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Proteomics-informed analysis of drug disposition in the human liver and small intestine

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Abstract
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Orally administered drugs are absorbed in the intestine and generally metabolized in the liver. Therefore, understanding factors determining drug distribution and elimination in these tissues is important. This thesis aimed at using mass spectrometry (MS)-based proteomics and functional studies to better understand *in vitro* model systems used for drug clearance predictions. Further, it aimed at understanding the changes in drug disposition caused by obesity and gastric bypass surgery (GBP).

The study was initiated by investigating factors influencing MS-based protein quantification by comparing results from different proteomics methods, and by studying protein distribution during subcellular fractionation. The largest variability in protein quantification was ascribed to insufficient enrichment from subcellular fractionation, most likely due to collection of the majority of the proteins in the initial fraction of the fractionation protocols.

Proteomics and metabolic activity analyses were then used to investigate differences in intrinsic clearance from two commonly used *in vitro* systems, human liver microsomes and hepatocytes. For some compounds, the faster microsomal metabolism could be explained by a higher available unbound drug concentration and CYP content in the microsomes as compared to in the hepatocytes.

Next, inter-individual protein expression variability in human liver and jejunum was explored. This showed that proteins covered a wide inter-individual variability spectrum, in which proteins with low variabilities were associated with essential cellular functions, while many proteins with high variabilities were disease-related.

Further, the effects of obesity, GBP, and weight loss on the proteomes of human liver and jejunum were analyzed. After GBP and subsequent weight loss, patients showed lower levels of jejunal proteins involved in inflammatory response and drug metabolism.

Finally, proteomics data from patients with and without obesity was combined with parameters from *in vitro* transport kinetics, and a mechanistic model to predict drug disposition was developed. The model successfully predicted rosuvastatin plasma concentrations in the patients.

In conclusion, this thesis has provided insights into factors influencing protein quantification and function *in vitro*. Furthermore, this thesis demonstrates how proteomics contributes to improved understanding of inter-individual and physiological differences, and how it can be used for *in vitro-in vivo* scaling of drug clearance.

Keywords: proteomics, protein concentration, drug disposition, drug transport, drug metabolism, human small intestine, human liver, human hepatocytes, human liver microsomes, inter-individual variability, drug clearance, obesity, prediction model

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When life gives you lemonade, make lemons. Life will be all like "What?!"

Phil Dunphy

List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.

I Wegler, C., Gaugaz, F.Z., Andersson, T.B., Wiśniewski, J.R., Busch, D., Gröer, C., Oswald, S., Norén, A., Weiss, F., Hammer, H.S., Joos, T.O., Poetz, O., Achour, B., Rostami-Hodjegan, A., van de Steeg, E., Wortelboer, H.M., Artursson, P. (2017) Variability in Mass Spectrometry-based Quantification of Clinically Relevant Drug Transporters and Drug Metabolizing Enzymes. *Molecular Pharmaceutics*, 14(9):3142-3151

II Wiśniewski, J.R., Wegler, C., Artursson, P. (2016) Subcellular fractionation of human liver reveals limits in global proteomic quantification from isolated fractions. *Analytical Biochemistry*, 15(509):82-88

III Wegler, C., Matsson, P., Krogstad, V., Urdzik, J., Christensen, H., Andersson, T.B., Artursson, P. Bridging differences in CYP activity between donor-matched human liver microsomes and hepatocytes. (*In manuscript*)

IV Wegler, C.[1], Ölander, M.[1], Wiśniewski, J.R., Lundquist, P., Zettl, K., Åsberg, A., Hjelmesæth, J., Andersson, T.B., Artursson, P. Global expression variability of proteins across and within human tissues. (*Submitted*)

V Wegler, C., Robertsen, I., Wiśniewski, J.R., Hjelmesæth, J., Åsberg, A., Andersson, T.B., Artursson. P. Effects of obesity and weight loss on global protein expression in human liver and jejunum. (*In manuscript*)

VI Wegler, C., Prieto Garcia, L., Klinting, S., Robertsen, I., Wiśniewski, J.R., Hjelmesæth, J., Åsberg, A., Jansson-Löfmark, R., Andersson, T.B., Artursson, P. Proteomics-informed prediction of rosuvastatin clearance in donors with and without obesity. (*In manuscript*)

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Abbreviations

AAFD	Absolute average fold difference
AAFE	Absolute average fold error
ABC	ATP-binding casette
A_{cell}	Drug amount in cell
ADME	Absorption, distribution, metabolism, and excretion
AFD	Average fold difference
AFE	Average fold error
A_{inc}	Amount of total protein in the incubation
AUC	Area under the curve
BCA	Bicinchoninic acid
BMI	Body mass index
CL	Clearance
C_{max}	Peak plasma concentration
C_{medium}	Drug concentration in the medium
$C_{u,cell}$	Intracellular unbound drug concentration
$C_{u,medium}$	Extracellular unbound drug concentration
CYP	Cytochrome P450
DDA	Data-dependent acquisition
E_{cell}	Protein concentration of transporter in cell system
E_{liver}	Protein concentration of transporter in liver
ER	Endoplasmic reticulum
FASP	Filter-aided sample preparation
FBS	Fetal bovine serum
FDR	False discovery rate
$f_{u,cell}$	Intracellular fraction unbound
$f_{u,hom}$	Fraction unbound in cell homogenate
$f_{u,medium}$	Fraction unbound in medium
GO	Gene ontology
GBP	Gastric bypass surgery
HEK293	Human embryonic kidney 293 cells
HomPPGL	Homogenate protein per gram liver
k	Slope from linear regression
K_m	Michaelis-Menten constant
Kp	Total intracellular drug accumulation ratio
Kp_{uu}	Intracellular unbound drug accumulation ratio
LC-MS/MS	Liquid chromatography-tandem mass spectrometry

MED-FASP	Multi-enzyme digestion filter-aided sample preparation
MPPGL	Microsomal protein per gram liver
MRM	Multiple reaction monitoring
M_w	Molecular weight
NADPH	Nicotinamide adenine dinucleotide phosphate
PCA	Principal component analysis
P_{cell}	Protein amount in cell
P_{hom}	Protein concentration in hepatocyte homogenate
PLS	Partial least squares
PM	Plasma membrane
r	Pearson's correlation coefficient
r_s	Spearman's rank correlation coefficient
RYGB	Roux-en-Y gastric bypass
SDS	Sodium dodecyl sulfate
SLC	Solute carrier
$T_{1/2}$	Terminal half-life
TPA	Total protein approach
UGT	Uridine-diphosphate-glucuronosyltransferase
V_{cell}	Cellular volume
V_{inc}	Incubation volume
V_{max}	Maximal transport or metabolic rate

Introduction

Oral drug administration is the most common administration route, in which the drug is delivered via the mouth through the gastrointestinal tract. During this route, several steps determine how much drug reaches the target to exert its effect. A solid oral dosage form (e.g., tablet) needs to disintegrate to finer particles from which the drug can dissolve. Once dissolved, the drug can be absorbed by the epithelium of the gastrointestinal tract (e.g., small intestine) and delivered to the target tissue via the systemic blood circulation. Most drugs are then eliminated from the body after being metabolized in the liver and excreted to the bile or urine. Thus, apart from the activity of the drug, its absorption, distribution, metabolism, and excretion (ADME) greatly influence its overall efficacy.

To estimate the efficacy of a drug based on its ADME properties, it is important to understand the factors influencing the drug disposition. These factors involve the physico-chemical properties of the drug, as well as the physiological barriers that the drug can encounter in the human body. Different types of proteins, such as drug-transporting proteins and drug-metabolizing enzymes, can alter the transport into cells and the metabolism of the drugs.

Transport and metabolism of drugs can be investigated using *in vitro* systems containing specific drug transporters or enzymes, which can help to determine drug activity and pharmacokinetics. To further improve the understanding of drug disposition in the human body, the data obtained *in vitro* can be extrapolated to the *in vivo* situation, using physiologically relevant prediction models. Differences between the *in vitro* system and the *in vivo* situation can be adjusted for by measuring the protein expression profiles (proteome) in the *in vitro* system, as well as in tissues involved in drug disposition.

The human small intestine

The small intestine is the longest part of the gastrointestinal tract, with a mean length of 5 m in humans, and is the main absorption site for nutrients and orally administered drugs.[1,2] The small intestine is divided into the duodenum, the jejunum, and the ileum. The duodenum measures the first 20-30 cm from the stomach and receives partly digested food, which is further chemically digested with the aid of pancreatic enzymes and bile from the gallbladder and liver. The jejunum begins at the distal end of the duodenum, at the ligament

of Treitz, and covers approximately 2 m of the small intestine. The jejunum absorbs nutrients that are released from the digestion and not absorbed by the duodenum. The ileum reaches approximately 3 m and constitutes the final part of the intestine, in which nutrients keep getting absorbed.[3] Throughout the intestine, the wall is composed of four distinct layers: the outermost layer of connective tissue (known as serosa), the muscle layer (muscularis propria), the submucosa, and the inner layer (mucosa).[4]

The highly absorptive nature of the small intestine is helped by a large surface area, which is enhanced by circular folds of the submucosa/mucosa (plicae circulares; Figure 1a.). The folds are further covered by finger-like projections called villi (Figure 1b), which in turn are lined with a layer of absorptive epithelial cells with tubular extensions of the apical membrane called microvilli (Figure 1c). The mucosal layer consists of the epithelial cells overlaying blood vessel-rich connective tissue (lamina propria) that in turn rests on a layer of smooth muscle (muscularis mucosa).[4] The lamina propria is also rich in immune cellular components such as lymphocytes, and plays a role in intestinal inflammation.[5] The epithelial cells are derived from stem cells with high proliferative rate, located in the crypts of the villi, and consist of highly absorptive enterocytes, and three types of secretory cells (enteroendocrine cells, Paneth cells, and goblet cells).[6] The enterocytes comprise over 80% of the epithelial cells and are rich in proteins important for absorbing and digesting different types of nutrients.[7] Importantly, epithelial cells form tight junctions (zonula occludens) with neighboring cells, acting as a barrier for extracellular substances, such as microorganisms and xenobiotics.[8]

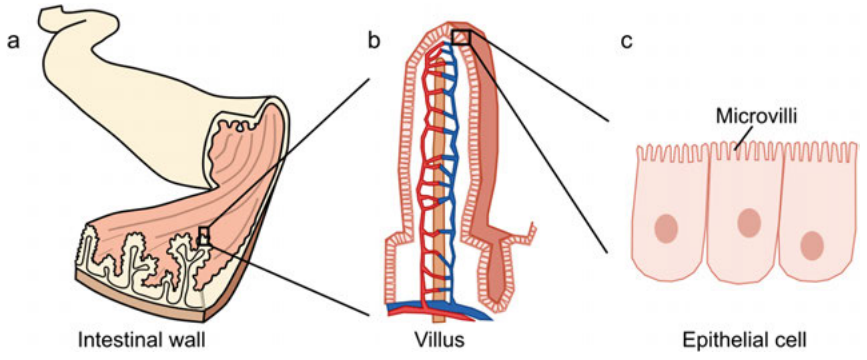

Figure 1. Microstructure of the small intestine. To increase the absorptive nature of the small intestine the surface area is enhanced by (**a**) circular folds (plicae circulares) of the inner layers (submucosa/mucosa), (**b**) finger-like projections (villi) lined with epithelial cells that have (**c**) tubular extensions of the apical membrane (microvilli).

The human liver

The liver is the largest internal organ in the human body, with a mean weight of 1500 g (approximately 2% of the total body weight), and plays an important role in detoxification and elimination of drugs and other xenobiotics from the blood.[9,10] The liver is closely connected with the gastrointestinal tract – both the stomach, at the gastrohepatic ligament, and the duodenum, through the hepatoduodenal ligament and porta hepatis containing the common bile duct.[11] The connection to the small intestine enables a recycling of substances from the liver to the bile, through the intestine and back to the liver, known as enterohepatic recirculation.[12] The liver is a highly vascularized organ, receiving up to 25% of the total cardiac output via the hepatic artery and the portal vein (that carries nutrients from the gastrointestinal system).[11] The liver is divided into structural units called lobules, which are composed of chords of liver cells organized around branches of the hepatic artery, portal vein, and bile ducts (Figure 2). Blood from the two different sources combines into small vessels creating a sinusoidal network that provides the liver cells with oxygen- and nutrient-rich blood that subsequently drains into the central vein.[13]

The liver is comprised of hepatocytes, representing approximately 60% of the liver cells and 80% of the total liver volume, and non-parenchymal cells including stellate cells, sinusoidal endothelial cells, and Kupffer cells.[14] Hepatocytes are epithelial cells responsible for the majority of the liver functions, including metabolic processes for the maintenance of glucose levels and the detoxification processes.

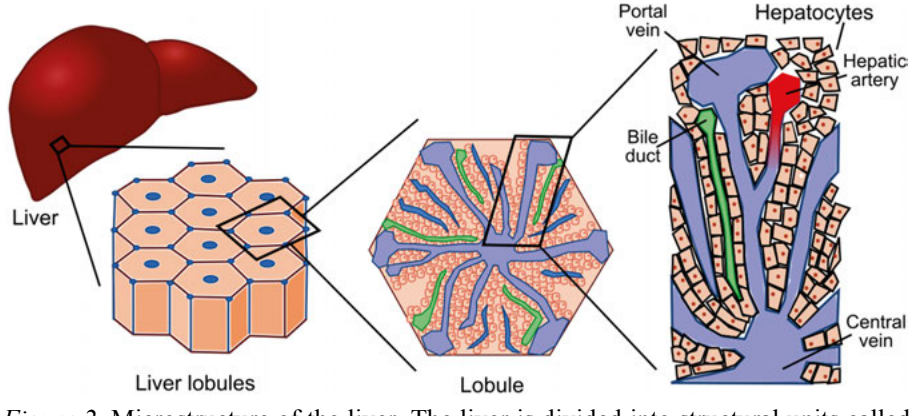

Figure 2. Microstructure of the liver. The liver is divided into structural units called liver lobules where liver cells, such as hepatocytes, are organized around branches of the hepatic artery, portal vein, and bile ducts, forming a sinusoidal network.

Studying biological processes at the protein level

Characterizing and understanding normal and disease-related cellular processes is one of the fundamental goals of molecular biology. DNA analysis (genomics) provides information about the genetic background, while studies of the mRNA levels (transcriptomics) give insight into gene expression features and regulation. Furthermore, measuring protein levels (proteomics) provides a picture of the functional units in the cell.[15-17] Since drug disposition is influenced by proteins such as transporters and enzymes, knowing their expression levels in different cells and tissues can greatly advance the understanding of the fate of drugs.

Immunoassays

Western blotting is one of the most widely used techniques in cell biology, and it is used to identify proteins extracted from cells. In this technique, proteins are separated based on their molecular weight through a polyacrylamide gel and transferred to a membrane with electrophoretic transfer.[18,19] Proteins are identified and semi-quantified from the membranes with the use of protein-specific antibodies. However, finding and producing antibodies with high specificity and low variability across batches is a major challenge for western blotting.[20,21] Furthermore, this technique is still limited to analyzing small sets of predetermined proteins at a time.

Radioimmunoassay (RIA) and enzyme-linked immunosorbent assay (ELISA) are examples of other antibody-based immunoassays that are used for qualitative or quantitative protein analysis. In these assays, a protein-specific antibody is immobilized on a surface that will 'capture' the protein of interest. The amount of protein is measured indirectly after displacement of radioactively labeled antigen bound to the antibody (RIA) or by enzymatic reactions from a secondary antibody (ELISA). These assays are commonly used in clinics as diagnostic tools largely due to their simplicity to use.[22,23] However, since they are also antibody-based, they share the same limitations of specificity and low throughput of the western blotting technique.

Mass spectrometry-based proteomics

With recent advances in technology, mass spectrometry (MS)-based proteomics has enabled the quantification of almost entire proteomes.[24] Proteomics strategies can be subdivided into top-down and bottom-up approaches, based on the method of protein characterization. Top-down approaches are used to study intact proteins, while bottom-up approaches are used for most quantitative or semi-quantitative proteomics analysis. In bottom-up proteomics, proteins are subjected to proteolytic digestion into peptides prior to analysis. After digestion, the peptides are typically separated with liquid chromatography

(LC) and analyzed with MS (LC-MS) to identify the sequences of the peptides, which are then assigned to the originating proteins (Figure 3).[15,16,25]

Sample preparation for bottom-up mass spectrometry-based proteomics

Protein levels in cells and tissues generally range over at least six orders of magnitude.[24,25] This makes it difficult to measure low abundant proteins, and it is sometimes necessary to perform enrichment before analysis. One method for protein enrichment is subcellular fractionation, in which proteins are typically separated with differential centrifugation by their organellar location based on different densities of the organelles.[26] Despite their shortcomings, such as unspecific loss of proteins in the preparation steps,[27] subcellular fractionation techniques are often used to enrich membrane proteins found in the endoplasmic reticulum (ER), such as enzymes, into vesicles that are commonly referred to as microsomes (see section 'Studying drug disposition *in vitro*)[28]. Subcellular fractionation can also enrich for transporters and receptors located at the plasma membrane.[26] Furthermore, cell surface proteins can be specifically targeted by chemical labeling, for which biotinylation is commonly used.[29] The enrichment of proteins of interest not only facilitates the measurement by increasing the protein levels, but also by reducing the sample matrix complexity that can interfere with the ionization of the MS.[30]

Further, there are a multitude of protocols for the protein extraction from cells, and subsequent protein digestion. The cellular protein content can be extracted using physical disruption, e.g., by homogenators, bead beaters, and sonicators. Other methods are reagent-based, including strong denaturants (e.g., urea), and ionic (e.g., sodium dodecyl sulfate (SDS)) or non-ionic detergents (e.g., Triton X-100).[31] Although efficient in extracting the proteins, many detergents are incompatible with MS since they suppress ionization.[32] This problem can be circumvented by removing the detergent and salts from buffers by gel electrophoresis separation, protein precipitation,[31] filter-aided sample preparation (FASP),[33] or the single-pot solid-phase-enhanced sample preparation (SP3).[34] In FASP, the proteins are kept on top of a filter and detergents are removed by repeated washes with highly concentrated urea buffer and centrifugation steps. The denatured and unfolded proteins on the filters are finally digested to peptides that pass through the filters for collection.[35] In SP3, the proteins are immobilized by hydrophilic interactions to carboxylate-coated magnetic beads, which allows the removal of unwanted reagents using organic solvents. Proteins are then digested and released from the beads by adding a water-based solvent.[34]

For protein digestion, the most commonly used proteolytic enzyme is the serine protease trypsin. Trypsin has a high cleavage specificity and efficiency, and cleaves the proteins at the C-terminal side of lysine and arginine amino acid residues, leaving peptides that are easily fragmented and analyzed by tan-

dem MS (MS/MS).[36] To improve the digestion efficiency, trypsin is often accompanied with lysyl endopeptidase (LysC) that cleaves the proteins after lysine, either as a mixture[37] or for sequential digestion.[38]

To increase the proteome coverage, the digest complexity can be reduced prior to the LC-MS proteomics analysis. For this purpose, digests can be prefractionated, in which peptides are separated based on e.g., hydrophobicity[39] or by their isoelectric point.[40] Furthermore, peptides can be enriched by immunoprecipitation from the digests.[41]

Targeted proteomics using mass spectrometry

Although the term proteomics was initially coined to describe the entire set of proteins in a cell or tissue,[42] the concept is also commonly used to describe the analysis of a limited number of proteins, i.e., targeted proteomics. In targeted proteomics, the pre-determined digested peptides are quantified as a proxy for protein abundance. Surrogate peptides are selected prior to analysis based on their capacity to be analyzed by MS (due to their physico-chemical properties) and their uniqueness to a specific protein (often referred to as proteotypic peptides). Since only one, or at best a few, peptide(s) are used for the quantification in targeted proteomics, proper selection is required to ensure accurate quantification. Special care should be taken to avoid hydrophobic transmembrane regions, known sites for post translational modifications, and sites for single nucleotide polymorphisms.[43]

Targeted proteomics almost always involves the use of isotopically-labeled standards that are spiked into the sample prior to analysis. The labeled standard can be introduced either in the form of protein prior to digestion, or more commonly as a peptide after digestion.[44] Peptide standards can be presented as single peptides, or as peptides concatenated into artificial proteins (QconCAT) that are cleaved to single peptides during the protein digestion step. By participating in the digestion step, the QconCAT strategy accounts for variation in the digestion efficiency.[45]

Since a limited number of proteins are quantified with targeted proteomics, the analyses times are relatively short, which is one of the reasons why targeted proteomics is commonly used to quantify specific proteins of interest for predictive pharmacokinetics.[46-49]

Global proteomics using mass spectrometry

Whereas both immunoassays and targeted proteomics can be used to study a limited number of pre-determined proteins, global proteomics is discovery-based and enables the analysis of entire proteomes. Global proteomics can be divided into two types of acquisition approaches: data-dependent acquisition (DDA) and data-independent acquisition (DIA). The DDA approach (also referred to as a ‘shotgun’ approach), analyses the peptides eluting from the LC and selects them for further fragmentation. This allows identification of peptide sequences by searching proteomics databases.[50] However, DDA is biased

since only a selection of peptide signals are analyzed every time, which can lead to an under-representation of peptides with low intensity. On the other hand, DIA (also known as SWATH) fragments all peptides in the sample by utilizing fragmentation windows, which reduces the focus on highly abundant peptides. Nonetheless, this unbiased approach leads to complex computational analysis and the requirement of vast peptide libraries unique for each analysis setup, which are generally acquired via a DDA approach.[51]

Shotgun proteomics can be used for qualitative analysis of proteins, for relative quantification using isobaric labeling (TMT or iTRAQ),[52] or quantification in a label-free manner. Label-free quantification methods include iBAQ, in which the protein intensities (corresponding to the sum of peptide intensities for that protein) are divided by the number of theoretically observed peptides,[53] and the total protein approach (TPA). In TPA, the protein concentration is calculated from the peptide intensities from the protein of interest divided by the sum of all peptide intensities in the sample, with the assumption that the summed peptide intensities in a sample represents the total protein content. Together with the molecular weight of the protein, the TPA can be used to determined specific concentrations in terms of mole per g total protein.[38] Recently, TPA was combined with TMT labeling, in which proteins in different samples were labeled with different isobaric tags and could thus be pooled.[54] This increases the throughput of the proteomics analysis, which can be useful since the deep proteome coverage requires relatively long analysis time.

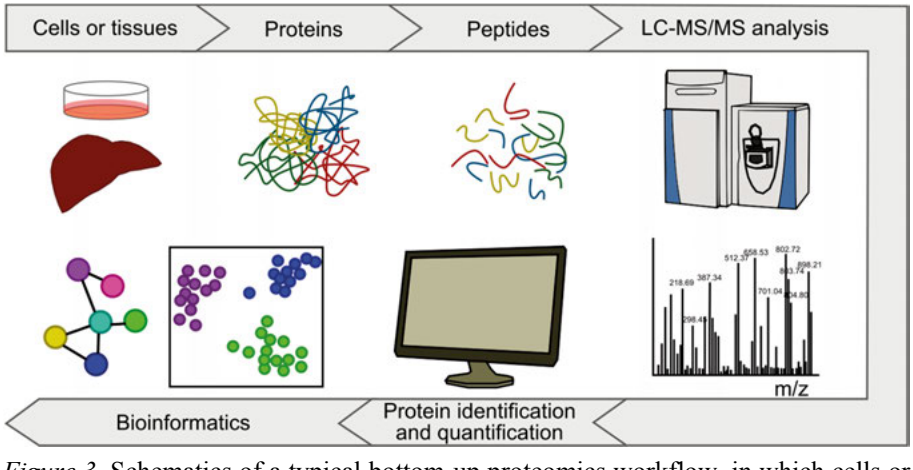

Figure 3. Schematics of a typical bottom-up proteomics workflow, in which cells or tissues are homogenized and lysed for protein extraction. Proteins are then enzymatically digested into peptides that are typically separated by liquid chromatography (LC), and analyzed with mass spectrometry (MS). The MS-data is processed for protein identification and quantification, which is used for bioinformatics analysis.

Label-free proteomics analysis has been used to understand the effect of the isolation process on the proteomes of hepatocytes,[55] study subpopulations of cells,[56] understand proteome changes related to cell health,[57] investigate differential protein expression across multiple tissues,[58] and study changes in the plasma proteome after weight loss.[59] Furthermore, label-free proteomics has proved useful for more specific protein quantification used in pharmacokinetic modeling.[60,61]

Drug disposition in jejunum and liver

Once a dissolved drug reaches the jejunum and liver, it will encounter several barriers before it can reach the blood stream. First, the lipophilic nature of the cellular membranes of both the jejunum and liver will limit the passage of large and hydrophilic compounds. In general terms, the drug can pass the membrane either passively by diffusion down the concentration gradient or facilitated by carriers. Second, once inside the cells, the drug can be actively transported back out of the cell or it can be metabolized.

Passive transfer across membranes

Drugs can pass through membranes (into or out of cells) by diffusion, which is the natural tendency for a molecule to move from a side with high concentration to another side with low concentration. Passive diffusion does not require any energy, and is driven by increased entropy in the system.[62] The extent to which a drug can move over cellular membranes by passive diffusion is determined by its molecular properties, such as size, lipophilicity, and ionization state.[63,64] To pass the epithelial barrier, the drugs can pass either between the cells (paracellular diffusion) or through the cells (transcellular diffusion). The paracellular pathway is regulated by tight-junction proteins connecting the epithelial cells and, for most drugs, only contributes to a small extent of the tissue absorption.[65,66] In contrast, the transcellular diffusion is the major pathway for absorption and tissue distribution of drugs.[67,68]

Carrier-mediated transport across membranes

In addition to passive diffusion, transport proteins that are integrated in the membrane can mediate the transport of drugs across the cell membrane. There are different mechanisms for carrier-mediated transport. Passive facilitated diffusion (by equilibrating transporters) does not require energy, while active transport requires energy to move a drug against the concentration gradient.[69] Active transporters are categorized as either primary or secondary depending on the source of energy, where the primary transporters utilize the energy from ATP hydrolysis, and secondary transporters often use ion gradients generated

by ATP-dependent primary transporters.[70] Depending on the location and direction of the transporter protein, the transporter will mediate the transfer of the drug into (influx or uptake) or out of (efflux) the cells, thus increasing or limiting the permeation of the drug through the tissue.

Enterocytes contain uptake and efflux transporters at the membrane of the intestinal lumen. These transporters regulate the transport of drugs from oral administration into the cell. Furthermore, transporters at the enterocyte membrane facing the blood regulate the amount of drug that reaches the portal vein (Figure 4). Similarly, the polarized hepatocytes contain uptake and efflux transporters at the basolateral membrane (facing the blood) that regulate the drug transport into the cell, and also biliary efflux transporters at the canalicular membrane (Figure 4).[71]

The two major superfamilies of uptake and efflux transporters, solute carrier (SLC) and ATP-binding cassette (ABC), consist of more than 400 membrane transporters where many are important for drug disposition.[71] Since the transporters are heterogeneously expressed in different tissues, their role in drug disposition can vary across organs.[72,73] Therefore, different panels of important drug transporters have been suggested for pharmacokinetic and drug interaction studies using enterocytes and hepatocytes (Figure 4).[71,74]

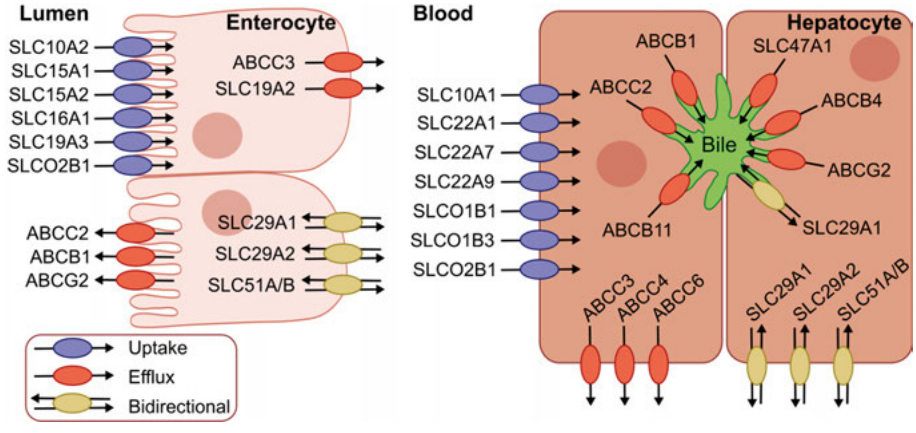

Figure 4. Localization and direction of important drug transporting proteins in enterocytes and hepatocytes.[71]

Metabolism of drugs

Once inside the cell, metabolic enzymes can metabolize drugs into more water-soluble metabolites, generally to increase elimination.[75] Drug metabolism is often divided into two steps, phase I metabolism, where the drug is converted to a more polar metabolite, and phase II metabolism, in which the polar metabolite can be conjugated with a charged group to facilitate urinary excretion.[76] The major phase I drug-metabolizing enzymes are cytochromes P450s (CYP3A4, CYP3A5, CYP2J2, CYP2E1, CYP2D6, CYP2C19, CYP2C8,

CYP2C9, CYP2B6, CYP2A6, and CYP1A2) and the major phase II enzymes are UDP-glucuronosyl transferases (UGT1A1, UGT1A3, UGT1A4, UGT1A6, UGT1A8, UGT1A10, UGT2B4, and UGT2B7).[77,78] Although the intestine expresses many drug metabolizing enzymes, the intestinal metabolism is considered to play a limited role in systemic drug metabolism.[79,80] Instead, drug metabolism occurs mainly in the liver due to the many highly expressed metabolizing enzymes in the hepatocytes.[10]

Altered functions of liver and jejunum by obesity

Humans with a body mass index (BMI) over 30 kg/m^2 are considered to suffer from obesity.[81] Obesity is a growing global health problem, since it increases the risk for cardiovascular disease and diabetes.[81] Obesity is not only associated with increased fat mass, but also with other physiological changes, such as increased blood volume, fat infiltration in the liver, and increased gut wall permeability, all of which could alter drug disposition.[82] The molecular mechanisms behind altered drug disposition in patients with obesity is not fully established.[83] For some drugs, changes in the volume of distribution affect the drug clearance, while the clearance of other drugs are mainly changed due to altered CYP expression in patients with obesity.[83-85]

Weight loss is the primary treatment of obesity and when diet, exercise, and pharmacotherapy are not enough, patients with obesity can undergo bariatric surgery. One well-established bariatric surgery procedure is the Roux-en-Y gastric bypass (RYGB), in which a small pouch is created from the proximal portion of the stomach that is attached directly to the jejunum, bypassing part of the stomach, duodenum, and a small part of the jejunum.[86,87] It is not fully established how drug disposition is affected by gastric bypass surgery, but factors such as reduction of absorptive sites, alterations in the mucosa, and hyperplasia (thickening of the intestinal wall) could contribute to alterations in drug disposition.[88,89]

Studying drug disposition *in vitro*

In drug development the mechanisms of intestinal and hepatic drug disposition must be determined in order to understand drug-drug interactions and alterations in activity due to genetic variation of transporters and enzymes. For this purpose, *in vitro* tools have proved useful for evaluating, for example, intestinal absorption, and hepatic distribution and metabolism by establishing the pharmacokinetics of drug transport and metabolism.[90,91] To determine the pharmacokinetics of drugs, the relationship between the rate of metabolism or transport (v) and the substrate concentration (S) can be investigated by the Michaelis-Menten model (see eq. 6 in Methods, section ‘Uptake transport in

cell lines').[92,93] The two Michaelis-Menten parameters V_{max}, which is a measure of the maximal rate of the reaction, and K_m, which is the substrate concentration that gives a reaction rate that is half of V_{max}, are typically determined using this model. These *in vitro* parameters are in turn often used to calculate the 'intrinsic clearance' (CL_{int} = v/S), as a measure of the metabolic or transport activity. CL_{int} is usually approximated to V_{max}/K_m, provided that the substrate concentration is markedly less (< 10%) than the K_m-value.[94]

The Michaelis-Menten parameters are typically determined by measuring the change in rate of activity of the transport or metabolism with increasing substrate concentration. For drug transport, the activity rate refers to the transfer across the cell membrane, while for drug metabolism, the activity rate refers to the conversion of the drug to its metabolite, and can be determined from the metabolite formation rate.[95,96] If the drug metabolism pathway is unknown, drug clearance can be measured by determining the rate of which the drug disappears as it is being metabolized (known as the substrate depletion method). This approach considers the contribution of several metabolic pathways, but shows limitations for slowly metabolized compounds where the changes can be too small to observe.[97,98]

Drug transport

When investigating the transport of drugs, *in vitro* systems are typically used to measure the flux of a drug from one side of a membrane to another. Drug transport can be investigated using Caco-2 cells originating from a human colon carcinoma[99] or other cell lines. The contribution of specific transporters to the disposition of drugs can be investigated using cell lines overexpressing particular transporters. Typical cell lines that are used as overexpression systems are Chinese hamster ovary (CHO), Madin-Darby canine kidney (MDCK), and human embryonic kidney 293 (HEK293) cells.[48,100,101]

HEK293 cells are commonly used for uptake studies. In such assays, cells, with or without an overexpressed uptake transporter, are exposed to drugs during a certain time period. The drug accumulation inside the cells is determined and used as a measure for uptake activity.[48,60,100,102]

To study the contribution of specific efflux transporters, inverted vesicles containing efflux transporters can be used. In such vesicles, the efflux transporter is directed into the vesicles instead of out towards the medium, which enables active transport of the drug from the incubation medium into the vesicles, where it can be measured in a similar manner to the uptake transport.[61,103]

A more holistic approach for studying drug transport is by using hepatocytes. Since hepatocytes express all hepatic uptake and efflux transporters (and hepatic drug metabolizing enzymes) they are considered the gold standard for studies of hepatic drug disposition *in vitro*.[104] Hepatocytes are typically isolated from liver tissue by enzymatic digestion of the connective tissue.[105]

Different configurations can be used for the transport studies, with the simplest approaches consisting of keeping the hepatocytes in suspension or cultured as a conventional monolayer on collagen-coated plates.[102,106] However, in such configurations, hepatocytes tend to dedifferentiate and eventually lose the ability to perform liver-specific functions, including drug transport and metabolism.[107] To overcome this problem and better resemble the *in vivo* situation, the hepatocytes can be cultured under a second layer of collagen (representing extracellular matrix in the liver), in a so-called sandwich culture format. This configuration promotes the reformation of bile canaliculi, enabling the study of biliary efflux.[108,109] For longer-term studies, hepatocytes can be cultured in the form of spheroids, in which hepatocytes are let to self-aggregate. This three-dimensional architecture allows cell-cell contact on all sides, with even more resemblance to the situation in the liver.[110,111]

Drug metabolism

Different *in vitro* systems can be used to investigate the metabolism of a drug. For instance, recombinant enzymes, typically purified from *Escherichia coli*, are readily available and useful for identifying the contribution of specific enzymes to the drug metabolism.[112,113] However, since the drug is in contact with only one or a few enzymes, the usefulness of recombinant enzymes is limited for determining drug clearance, where more complex *in vitro* systems are favorable.

One commonly used *in vitro* system for determining drug clearance is liver microsomes. Microsomes are isolated from tissues or cells by differential centrifugation.[26] Although microsomes are a well-established concept, there is a multitude of protocols available for the isolation process with varying number of steps, centrifugation speeds, and times, which can influence the reproducibility of this *in vitro* system. There is a consensus of removing cell debris and nuclei in an initial low-speed centrifugation step, but this step varies greatly between protocols, with centrifugation speeds varying from 560 to 20 000 × g.[26,114-121] In some protocols, mitochondria and lysosomes are further separated (3 000 to 20 000 × g)[26,114,115,117,120] prior to the isolation of the microsomal fraction that is typically obtained after ultracentrifugation with 100 000 to 110 000 × g.[116,118,120,121] The microsomal fraction is considered to be vesicles derived from the ER, giving an enrichment of metabolic enzymes located in the ER, such as CYPs and UGTs.[120] Since microsomes are not composed of intact cells, the activity of CYPs and UGTs require the addition of the cofactors NADPH and UDPGA.[122,123] Microsomes have proven useful in pharmacokinetic and drug-drug interaction studies, but are limited by the activated pathways in the assay (by the addition of cofactors).[97,124-126] On the other hand, hepatocytes contain a complete set of the hepatic proteins important for drug disposition, making them more relevant for *in vivo* comparisons. In metabolic activity assays, the various configurations of hepatocytes described above are

common.[127] In assays with hepatocytes, the contribution of both transport and metabolism on drug clearance can be studied, giving information about overall drug disposition processes.[96,128,129]

Intracellular unbound drug concentrations

When studying drug disposition, it is important to consider that only the unbound drug that reaches the intracellular target can exert its effect. Multiple factors can limit this parameter, including drug binding and cellular disposition. Drugs can bind to proteins in the blood, cells, and incubation medium, or to phospholipids of the cell membrane.[130] Drug transport into a cell can be limited by its physico-chemical properties, drug transporters, and metabolizing enzymes. Drug binding and cellular concentrations can be accounted for with the intracellular unbound drug accumulation ratio (Kp_{uu}), in which the drug-specific unbound fraction is combined with the cellular accumulation of the drug.[131-136] The unbound fraction of a drug is typically determined from equilibrium dialysis where partitioning of a drug between two sides of a dialysis membrane (one side with cell homogenate and drug, and one side with buffer) is determined. Further, the cellular accumulation ratio (Kp) of a drug is often assessed from cell assays, in which the drug concentration equilibrium between cells and medium is determined.

The unbound intracellular drug concentration has been used to bridge differences between biochemical and cellular potency assays[134], to improve predictions of time-dependent CYP inhibition[133], and as a scaling factor to explain differences in CYP enzyme inhibition in microsomes and hepatocytes.[136]

Scaling of pharmacokinetics from *in vitro* to *in vivo*

A common aim of studying drug disposition *in vitro* is to extrapolate the data to the *in vivo* situation. Accurate predictions of drug disposition are important in drug development for understanding drug-drug interactions and clearance alterations caused by changes in expression or activity of transporters and enzymes due to polymorphic genotypes or physiological differences.[137] For this purpose, pharmacokinetic models can be created. Typical pharmacokinetic models range in complexity from being empirical (e.g., clearance predictions), to semi-mechanistic (using compartments of tissues of interest), to physiologically based models (PBPK) using a broader understanding of the physiology of the body.[137] Modeling approaches can be separated into top-down, in which models are built based mainly on observed clinical data, bottom-up, using *in vitro* parameters that are scaled to *in vivo* with physiological scaling factors (Figure 5), and middle-out, using *in vitro* data when available and simulating remaining parameters from *in vivo* data.[138]

Commonly, drug clearance determined in microsomes and hepatocytes is scaled to *in vivo* directly by adjusting the clearance with the amount of microsomal protein and number of hepatocytes in the liver together with the liver weight (referred to as microsomal protein per gram liver, MPPGL, and hepatocellularity).[128,129,139,140] This type of scaling assumes that the activity of the microsomes and hepatocytes correspond to that *in vivo*. However, altered enzyme levels in microsomes due to the fractionation process, and changes in protein expression during hepatocyte culturing can give rise to poor predictions of *in vivo* clearance.[27,141]

For microsomal scaling, a MPPGL of 45 mg microsomal protein per g liver was introduced in the early 1990s and has been frequently used for scaling microsomal clearance to *in vivo*.[97,128,129,139] This scaling factor was initially summarized from rat microsomes and its accuracy has been questioned as it does not account for variability in protein recovery during the microsomal preparation.[125,142-145] Furthermore, the scaling factor is based on total microsomal protein amount assuming that the microsomes mainly contain proteins from the ER, with enrichment of CYPs and UGTs. Similarly, the commonly used value for hepatocyte scaling, a hepatocellularity of 120 million cells per gram liver, originates from rat hepatocytes.[142,146] Assuming that the hepatocellularity is transferrable across species, this value still does not consider variability from different hepatocyte batches.[145] An emerging approach to correct for these alterations is to incorporate protein concentrations from the *in vitro* system and tissue into the prediction model (Figure 5).[147]

The approach of correcting for protein expression has been readily used for scaling transport activity data obtained from transfected cell lines and membrane vesicles. Although these *in vitro* systems differ considerably from the *in vivo* situation, the assumption of a linear correlation between activity and protein expression allows for scaling using protein expression from *in vitro* and *in vivo*.[48,49,60,148-150]

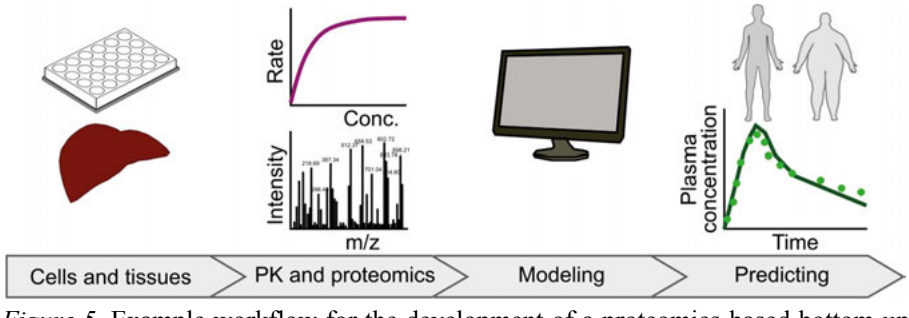

Figure 5. Example workflow for the development of a proteomics-based bottom-up prediction model. Pharmacokinetic (PK) parameters determined from cell assays can be scaled to the *in vivo* situation with proteomics data from cells and tissues. This information can be used in mechanistic models to predict plasma drug disposition.

Aims of the thesis

The overall aim of this thesis was to use mass spectrometry-based proteomics and functional studies of drug transporters and metabolizing enzymes to better understand *in vitro* model systems that are used for prediction of drug clearance. Further, it aimed at understanding the changes in drug disposition caused by obesity and gastric bypass surgery.

The specific aims were:

- to investigate factors influencing mass spectrometry-based protein quantification by comparing different sample preparation workflows and proteomics methods (Paper I), and to understand how proteins distribute during subcellular fractionation (Paper II)

- to understand differences in drug clearance from the two *in vitro* systems liver microsomes and hepatocytes, as an effect of protein content and intracellular unbound drug concentrations (Paper III)

- to investigate inter-individual protein expression variability within human liver and jejunum (Paper IV)

- to investigate how obesity, gastric bypass surgery, and weight loss affect the proteomes of human liver and jejunum (Paper V)

- to develop a proteomics-informed mechanistic model from *in vitro* model systems for predicting drug plasma distribution of rosuvastatin in patients with and without obesity (Paper VI)

Methods

Proteomics analysis

Bottom-up proteomics analysis was used in Papers I-VI. The general bottom-up proteomics workflow was initiated with a sample preparation step where proteins were isolated from the tissues or cells, and subsequently enzymatically digested to peptides. The peptides were then analyzed either with a targeted or global liquid chromatography-tandem mass spectrometry (LC-MS/MS) based proteomics approach.

Sample preparation

Human liver and jejunum biopsies or cells were homogenized in lysis buffer containing a high concentration of SDS, and proteins were denatured at high temperature. Samples were prepared for proteomics analysis using the filter-aided sample preparation (FASP)[33] or multi-enzyme digestion FASP (MED-FASP)[151] protocols, in which proteins were digested with trypsin alone (FASP), or sequentially digested with LysC and trypsin (MED-FASP). Protein and peptide amounts were determined based on tryptophan fluorescence.[152]

Liquid chromatography-tandem mass spectrometry-based targeted proteomics

In Papers I and VI, peptides obtained from the FASP protocol were subjected to LC-MS/MS-based targeted proteomics. With this approach, specific proteins were quantified using isotope-labeled and unlabeled peptide standards, with unique amino acid sequences identical to sequences from the protein of interest. The peptides were analyzed by tandem mass spectrometry on a triple quadrupole instrument (QTRAP 6500) with selective monitoring of mass-to-charge transitions (MRM mode) corresponding to the targeted peptide. Peaks from each transition were integrated with the MultiQuant software. Each protein was quantified by the relationship of the peak area of the unlabeled target peptide and the peak area of the spiked-in labeled peptide standard obtained from three mass transitions.[153]

Liquid chromatography-tandem mass spectrometry-based global proteomics

In Papers II-VI, LysC and tryptic peptides obtained from the MED-FASP protocol were subjected to LC-MS/MS-based global, label-free, proteomics. The peptide mixtures were analyzed on a high-resolution Q Exactive HF or Q Exactive HF-X mass spectrometer. In the global proteomics approach, survey and MS/MS scans enable analysis of most of the peptides in the sample. Proteins were identified from the obtained MS data using the MaxQuant software,[50] combining the spectra of the LysC and tryptic fractions and searching them against a theoretical human proteome database (Uniprot accession number: UP000005640). Protein concentrations were calculated using the total protein approach (TPA), with the assumption that the sum of all peptide intensities in a sample represents the total protein content.[38] With this assumption, the protein concentration (mol/g total protein) can be calculated by dividing the intensity of peptides from a specific protein, taking into account its molecular weight, by the sum of all peptide intensities in the sample:

$$\text{Protein concentration} = \frac{\text{MS signal}}{\text{Total MS signal} \times M_W} \qquad \text{(eq. 1)}$$

where 'MS signal' is the intensity of peptides from a specific protein, 'Total MS signal' is the total intensity of all peptides in the sample, and 'M_W' is the molecular weight of the protein of interest.

Bioinformatics analysis

Global proteomics datasets were filtered and processed with Microsoft Excel, R, or the Perseus platform.[154] Statistical analyses were performed using GraphPad Prism, version 7.03-8, or R. Principal component analysis (PCA) and partial least square (PLS) modeling were conducted in SIMCA (version 15.0.0.4783).

Proteins with significantly different concentration levels in the different sample types were identified using t-test with a permutation-based FDR calculation (implemented in Perseus)[154] or *limma* analysis.[155] Functional annotation clustering of significantly different proteins was performed with DAVID Bioinformatics Resources (version 6.8), based on gene ontology (GO) terms and KEGG pathways.[156] Proteins were searched against the human proteome using default settings, and annotation clusters were considered significant at enrichment scores above 1.3 (corresponding to P-values < 0.05).

Proteins were annotated with subcellular location from Uniprot (implemented in Perseus)[154], the 'Subcellular location data' from the Human Protein

Atlas,[157] or from the Prolocate database.[158] The Panther Classification System[158] was used to classify proteins in protein classes based on GO terms, searching against the human proteome using default search settings.

Subcellular fractionation

In Paper II, the distribution of proteins in different subcellular fractions after differential centrifugation was investigated. For this purpose, liver tissues were homogenized in sucrose buffer and the liver homogenate was fractionated by centrifugation pelleting. After each centrifugation step, pellets were collected and the remaining supernatant was subjected to another centrifugation step. Fractions were collected after centrifugation for (A) 10 min at 1 000 × g, (B) 10 min at 2 000 × g, (C) 10 min at 5 000 × g, (D) 10 min at 10 000 × g, and (E) 60 min at 21 000 × g. Finally, the supernatant was collected for analysis of soluble proteins (F).

Metabolic activity

In Paper III, drug clearance, or metabolic activity, was measured in isolated human hepatocytes and liver microsomes, two commonly used *in vitro* systems.

Hepatocyte isolation

Human hepatocytes were isolated from liver resections using a two-step collagenase perfusion technique[105] and were cryopreserved in medium complemented with 10% FBS. Prior to use, cells were thawed at 37 °C, and dead cells were removed by centrifugation at room temperature in density gradient medium. Thawed hepatocytes were resuspended to 1 million cells/ml in medium (pH 7.4) containing insulin, transferrin, selenium, dexamethasone, penicillin, and streptomycin.

Microsome isolation

Human microsomes were isolated from snap-frozen liver tissue pieces. Liver tissues were homogenized in sucrose buffer and the homogenate was subjected to differential centrifugation. The liver homogenate was first centrifuged for 10 min at 7 400 x g at 4 °C. The supernatant was then further centrifuged for 60 min at 104 000 x g at 4 °C. Human liver microsomes were obtained from the remaining pellet, which was resuspended in sucrose buffer. Microsomes were thawed on ice and diluted to 0.5 mg/ml in phosphate buffer

(pH 7.4) prior to use. Metabolic activity reactions in microsomes were initiated with 1 mM NADPH.

Metabolic clearance measurement

The hepatocytes and microsomes were incubated with probe drugs to monitor specific CYP enzyme activities: bupropion (CYP2B6), bufuralol (CYP2D6), omeprazole (CYP2C19), diclofenac (CYP2C9), and midazolam (CYP3A4). The metabolic activity assays were performed during 90 min at 37 °C while shaking, and aliquots were taken after 0, 5, 10, 15, 20, 30, 60, and 90 min. Reactions were stopped by the addition of acetonitrile, which also extracted intracellular drug compounds. Compounds were quantified as described in the section 'Drug quantification with liquid chromatography-tandem mass spectrometry'. Metabolic clearance of the respective compound was determined with a substrate depletion method.[97] The slope from the linear regression (k) from log percent remaining drug, and incubation time was used to calculate the metabolic clearance (CL_{int}):

$$CL_{int} = - k \times \frac{V_{inc}}{P_{inc}} \quad \text{(eq. 2)}$$

where 'V_{inc}' is the incubation volume, and 'P_{inc}' is the amount of total protein in the incubation, measured with the Pierce bicinchoninic acid (BCA) protein assay reagent kit according to the manufacturer's instructions.

Intracellular unbound drug concentration

In Paper III, the unbound drug accumulation ratio (Kp_{uu}) in hepatocytes was determined for the CYP probes mentioned above. Kp_{uu} was calculated as the ratio of intracellular unbound drug concentration ($C_{u,cell}$) and extracellular drug concentration ($C_{u,medium}$) using:

$$Kp_{uu} = \frac{f_{u,cell}}{f_{u,medium}} \times Kp \quad \text{(eq. 3)}$$

where '$f_{u,cell}$' and '$f_{u,medium}$' correspond to the intracellular and medium unbound drug fractions, respectively. Binding in the extracellular space was considered to be negligible ($f_{u,medium} = 1$) since the incubations were performed in buffer. Kp is the intracellular drug accumulation ratio calculated from:

$$Kp = \frac{A_{cell} / (V_{cell} \times P_{cell})}{C_{medium}} \quad \text{(eq. 4)}$$

where 'A_{cell}' is the drug amount in the cells, 'C_{medium}' is the drug concentration in the extracellular space, 'V_{cell}' is the cellular volume (6.5 µl/mg protein)[159],

and 'P_{cell}' is the protein amount in the cells, measured with the Pierce bicinchoninic acid (BCA) protein assay reagent kit according to the manufacturer's instructions.[132]

After 15 and 30 min incubations, an aliquot of cell suspension was collected upon which cells were separated from the medium by 5 min centrifugation at 100 × g at 4 °C. Drug compounds were determined in medium (C_{medium}) and cells (A_{cell}) as described in the section 'Drug quantification with liquid chromatography-tandem mass spectrometry'.

Intracellular drug binding ($f_{u,cell}$) was measured using dialysis, in which hepatocyte homogenate was spiked with the compounds and dialyzed for 4 h at 37 °C using a Rapid Equilibrium Dialysis device.[134] The intracellular unbound fraction ($f_{u,cell}$) was calculated according to:

$$f_{u,cell} = \frac{1}{D \times (1/f_{u,hom} - 1) + 1} \quad \text{(eq. 5)}$$

where D was used to correct for homogenate dilution and was calculated as $1/(V_{cell} \times P_{hom})$, and where '$P_{hom}$' is the protein concentration in the hepatocyte homogenate. The fraction of unbound drug in the cell homogenate ($f_{u,hom}$) was calculated as the ratio of the drug concentration in the receiving buffer chamber (C_{buffer}) and the drug concentration in the homogenate chamber (C_{hom}).

Uptake transport in cell lines

In Paper VI, hepatic transport-mediated uptake of rosuvastatin was assessed using HEK Flp-In-293 cells stably expressing either SLCO1B1, SLCO1B3, SLCO2B1, or SLC10A1.[60,160] Cells were incubated with varying concentrations of rosuvastatin for 2 min at 37 °C. Active transporter-mediated uptake was calculated by subtracting the accumulation in mock-transfected cells (passive diffusion) from the uptake in the transporter-transfected cells. An uptake curve was established with the initial uptake rate related to the substrate concentration and was fitted to the Michaelis-Menten equation:

$$v = \frac{V_{max} \times [S]}{K_m + [S]} \quad \text{(eq. 6)}$$

where 'v' is the uptake rate, 'V_{max}' is the maximal uptake rate, '[S]' is the substrate concentration, and 'K_m' is the substrate concentration at which the uptake rate is half of V_{max}. Passive clearance ($CL_{passive}$) of rosuvastatin was determined by linear regression analysis from the mock-transfected cells. Uptake clearance of rosuvastatin by each transporter ($CL_{transporter}$) was calculated by:

$$CL_{transporter} = \frac{V_{max}}{K_m} \quad \text{(eq. 7)}$$

Drug quantification with liquid chromatography-tandem mass spectrometry

Small molecular weight compounds were quantified with LC-MS/MS using a triple quadrupole mass spectrometer. For most compounds, a 2 min gradient with a linear increase of acetonitrile throughout the gradient was used to elute the compounds from a C_{18} column. Compounds were analyzed using tandem mass spectrometry with electrospray ionization. Warfarin was used as internal standard in all compound analysis.

Proteomics-informed *in vitro–in vivo* scaling of hepatic transport

In Papers I and VI, transporter-mediated uptake of atorvastatin and rosuvastatin, respectively, were assessed from $CL_{transporter}$ obtained from HEK293 cells expressing important liver uptake transporters (either SLCO1B1, SLCO1B3, SLCO2B1, or SLC10A1). The established *in vitro* clearance was used together with protein quantification to predict hepatic uptake clearance in one static (Paper I and VI) and one mechanistic model (Paper VI).

Prediction of hepatic uptake clearance

In Papers I and VI, the hepatic uptake clearance (CL_{uptake}) of atorvastatin and rosuvastatin, respectively was predicted. For this purpose, a static model was used to scale the *in vitro* transport capacity (V_{max}) to the *in vivo* situation with the use of protein concentrations *in vitro* (HEK293 cells) and *in vivo* (liver tissue), assuming that the maximal transport rate is directly related to protein concentration.[60,160,161] The following equation was used to scale the obtained clearance from each transporter ($CL_{transporter}$):

$$CL_{uptake} = \sum_{Transporters} CL_{transporter} \times \frac{E_{liver}}{E_{cell}} \times HomPPGL \qquad \text{(eq. 8)}$$

where 'E_{liver}' and 'E_{cell}' are the protein concentrations of the transporter in the liver tissue and cell system, respectively, and 'HomPPGL' is milligram of homogenate protein per gram of liver tissue (88 mg protein/g liver).[60] In Paper I, literature *in vitro* parameters (V_{max}, K_m, and protein expression in HEK293 cells) were used,[60] while *in vitro* parameters in Paper VI were determined as described above.

Mechanistic model for prediction of time-dependent drug disposition

Hepatic drug clearance *in vivo* changes over time as a result of altered blood and tissue concentrations of the drug. To capture these dynamic changes, a mechanistic model was built (Paper VI) to describe the time-dependent drug disposition of rosuvastatin in plasma by using predicted hepatic uptake clearance (CL_{uptake}) determined in HEK293 cells.

For this purpose, two types of mechanistic pharmacokinetic models were built: 1) a compartment model in which the oral dose was absorbed from the intestine into the liver, and subsequently distributed to the blood, assuming an instant mixing of blood from the portal and central vein:

$$\frac{dI}{dt} = -k_a \times I \quad \text{(eq. 9)}$$

$$\frac{dL}{dt} = \left(k_a \times I \times F + CL_{uptake} \times \frac{B}{V_B} + CL_{passive} \times \frac{B}{V_B}\right) - \left(CL_{passive} \times \frac{L}{V_L} + CL_{out} \times \frac{L}{V_L}\right) \quad \text{(eq. 10)}$$

$$\frac{dB}{dt} = \left(CL_{passive} \times \frac{L}{V_L} + CL_{efflux} \times \frac{L}{V_L}\right) - \left(CL_{passive} \times \frac{B}{V_B} + CL_{uptake} \times \frac{B}{V_B} + CL_{renal} \times \frac{B}{V_B}\right) \quad \text{(eq. 11)}$$

where 'I', 'B', and 'L' are the amount of drug in the intestine, blood, and liver, respectively, 'F'is the gastrointestinal absorption (0.5)[162], and 'k_a' is the absorption constant (0.244 h^{-1}, in-house estimate from AstraZeneca popPK-modeling). 'CL_{efflux}' is the active hepatic drug efflux from the blood to liver (0, assuming low active transport of rosuvastatin by basolateral efflux transporters),[48] 'CL_{out}' is the drug clearance from the liver (i.e., biliary and metabolic clearance; 1.23 µl/min/10^6 hepatocytes),[163] and 'CL_{renal}' is the drug clearance through the kidneys (13.6 L/h).[162] '$CL_{passive}$' is the passive clearance measured from the mock transfected cells and 'CL_{uptake}' is the total active uptake drug clearance between blood and liver from the four transporters (eq. 8). 'V_L' is the fractional liver volume (body weight × 0.021 µl)[164] and 'V_B' is the volume of distribution (0.227 L/kg estimated from [165,166]).

The second model 2) was an extension of model 1) but also included a gallbladder compartment to incorporate the enterohepatic recirculation to capture biphasic elimination of the drug:

$$\frac{dI}{dt} = (k_b \times G) - (k_a \times I) \quad \text{(eq. 12)}$$

$$\frac{dL}{dt} = \left(k_a \times I \times F + CL_{uptake} \times \frac{B}{V_B} + CL_{passive} \times \frac{B}{V_B}\right) - \left(CL_{passive} \times \frac{L}{V_L} + CL_{efflux} \times \frac{L}{V_L} + CL_{bile} \times \frac{L}{V_L} + CL_{met} \times \frac{L}{V_L}\right) \quad \text{(eq. 13)}$$

$$\frac{dG}{dt} = \left(CL_{bile} \times \frac{L}{V_L}\right) - (k_b \times G) \quad \text{(eq. 14)}$$

$$\frac{dB}{dt} = \left(CL_{passive} \times \frac{L}{V_L} + CL_{efflux} \times \frac{L}{V_L}\right) - \left(CL_{passive} \times \frac{B}{V_B} + CL_{uptake} \times \frac{B}{V_B} + CL_{renal} \times \frac{B}{V_B}\right) \quad \text{(eq. 15)}$$

where 'G' is the amount of drug in the gallbladder, 'k_b' is the biliary emptying rate (0.0013 min^{-1}),[167] 'CL_{bile}' is the hepatic clearance of drug to the gallbladder (1.23 µl/min/10^6 hepatocytes),[168] and 'CL_{met}' is the drug clearance by metabolism (3.52 L/h).[162]

Results and discussion

Variability in mass spectrometry-based protein quantification from different methodologies (Papers I and II)

In the first two papers, the effect of methodological differences on the outcome of bottom-up mass spectrometry-based proteomics analysis was investigated. Although bottom-up proteomics is widely used for protein quantification, the methodologies vary across laboratories, with differences in both sample preparation and approaches for quantification. When these studies were conducted, it had not yet been established to what extent variability in reported protein quantities was related to the choice of proteomics strategy.

In Paper I, the quantification of proteins important for drug disposition, in liver tissues from 10 donors, by different in-house proteomics approaches in six laboratories were compared. Three of the laboratories determined the membrane proteins in whole tissue digests while the other three laboratories analyzed the proteins after different types of subcellular fractionation. Protein quantification was performed using five different targeted approaches and one global, label-free approach. Largest variability in protein quantification was observed from the laboratories using subcellular fractionation processes. Therefore, in Paper II, the effect of subcellular fractionation on protein quantification was investigated by studying the distribution of proteins in different fractions obtained after sequential centrifugation of liver homogenate with centrifugal speeds increasing from 1 000 × g to 21 000 × g. In theory, proteins attached to different organelles will sediment at different centrifugal speeds based on density and thus separate to different subcellular fractions.[26] The analysis showed that many proteins are lost during the centrifugation processes leading to variable protein enrichment in the final fraction.

Protein concentrations quantified in six laboratories using different proteomics methodologies

In Paper I, major drug metabolizing enzymes (CYPs and UGTs) and drug uptake and efflux transporters (SLCs and ABCs) were quantified in the six laboratories. Both enzymes and transporters were quantified at comparable lev-

els in the whole tissue digests (Labs a, b, and c) with two-fold average difference across the three laboratories (Figures 6a and 6b). The higher complexity of the whole tissue digests lowered the reproducibility in two of the laboratories (intra-laboratory absolute average fold error of 1.8 in Labs a and b). This was confirmed by Lab c, which demonstrated high reproducibility after reduction of the whole tissue digest complexity by immunoprecipitation of selected peptides. Meanwhile, the comparable results from the laboratories using whole tissue digests showed that the label-free approach (Lab b) did not give rise to higher variability in quantification compared to the targeted approaches (Lab a and Lab c).

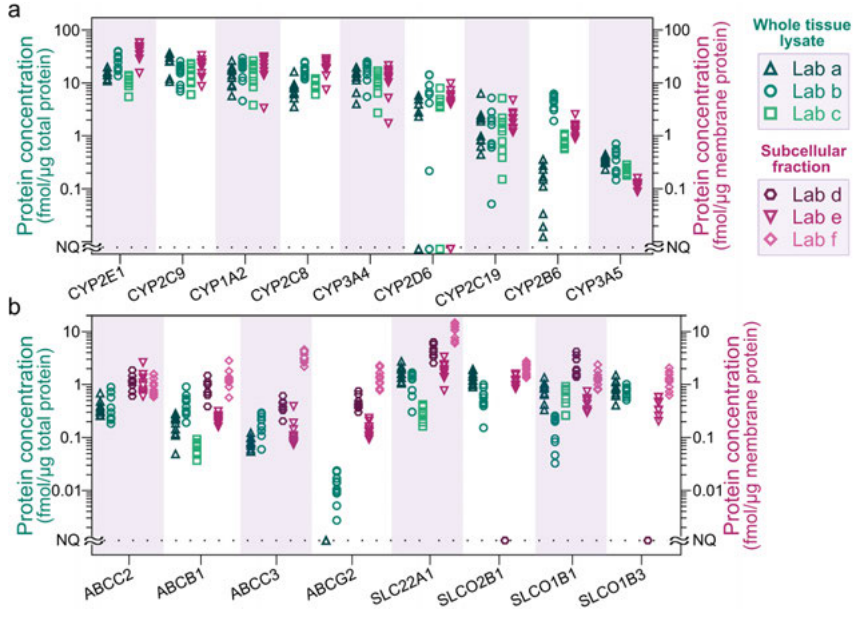

Figure 6. Comparison of liver protein quantification in ten donors determined in the different laboratories. (**a**) Important drug metabolizing enzymes. (**b**) Liver uptake and efflux transporters important for drug disposition. Protein levels from Labs a–c were quantified in whole tissue lysates (fmol/µg total protein) and protein levels from Labs d–f were quantified in concentrated subcellular fractions (fmol/µg membrane protein). NQ: not quantified, proteins could not be quantified with the applied methods.

Unexpectedly, the enzymes and transporters were quantified at comparable levels in the enriched subcellular fractions as to those in the whole tissue digests. For instance, the enzymes were quantified with only two-fold average difference (Lab e), and the transporters were quantified with four-fold average difference (Labs d, e, and f) compared with the whole tissue digests (Figures 6a and 6b). The low fold differences of proteins quantified in the isolated subcellular fractions compared with whole tissue digests demonstrate a low degree of enrichment and suggest a poor recovery of membrane proteins after

the subcellular fractionations in Labs d, e, and f. For instance, transporter proteins were only enriched up to three-fold in crude membrane fractions (Labs d and e), while full recovery of proteins during the crude membrane fractionation process is expected to give a four to six-fold enrichment, based on the assumption that the membrane fraction contributes 16 – 25% of the total cellular protein.[169-171] Further, transporters quantified in the isolated plasma membrane fractions (Lab f) were only enriched six-fold, while the expected enrichment would be 30 to 100-fold since the plasma membrane proteins are assumed to make up 1 – 3 % of the total cellular protein amount.[27,171,172]

Scaling protein levels from enriched subcellular fractions to whole tissue

To enable direct comparison of the quantified membrane proteins (transporters and enzymes) across all laboratories included in Paper I, the protein concentrations obtained in the isolated subcellular fractions (Labs d, e, and f) were scaled to those in whole tissue. This scaling was performed by assuming full recovery of proteins in the fractionation processes, and were based on the total protein amount (µg) in the subcellular fraction and the weight of tissue used for the isolation process (mg), together with the total protein amount per gram liver from unfractionated liver homogenate (88 µg/mg)[160]. In theory, based on the above assumptions, the corrected protein levels from the subcellular fractions should reach the same levels as those obtained directly from whole tissue digests.

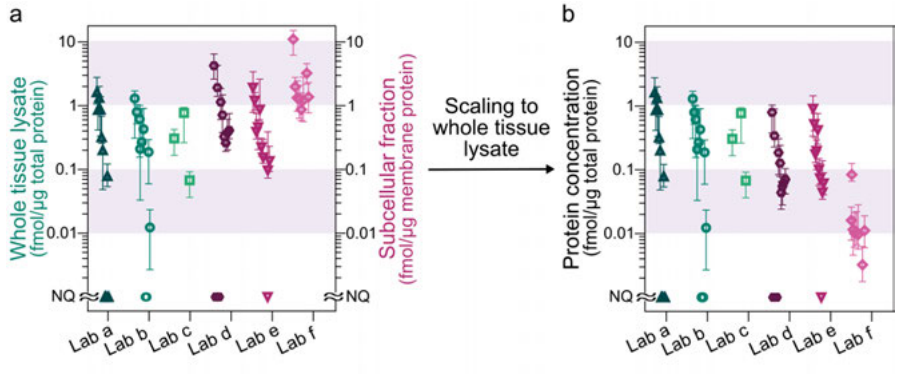

Figure 7. Comparison of transporter protein levels in (**a**) whole tissue digests (fmol/µg total protein; Lab a–c) and in concentrated subcellular fractions (fmol/µg membrane protein; Lab d–f), and (**b**) levels after correction to those in whole tissue lysate (fmol/µg total protein). Protein levels are displayed as geometric mean and minimum and maximum levels of ten donors. NQ: not quantified, proteins could not be quantified with the applied methods.

After scaling to whole tissue, it was evident that the lower recovery from the membrane isolation processes led to an underestimation of the protein concentrations (Figures 7a and 7b). The largest underestimation was obtained in Lab f, in which plasma membrane fractionation was used. In Lab f, the transporter proteins were consistently 10-fold lower compared to levels from the other methods ($P < 0.05$ based on a Mann-Whitney test).

Distribution of proteins during subcellular fractionation with differential centrifugation

To better understand the low enrichment and poor recovery of membrane proteins in the isolated subcellular fractions, we investigated the distribution of proteins across six different fractions obtained from differential centrifugation of liver homogenate (Paper II).

The proteomics analysis of the subcellular fractions in Paper II showed that proteins from different organelles were distributed across the six fractions. More than half of the total protein of the homogenate was obtained already in the first fraction, Fraction A (1 000 × g, 10 min). Meanwhile, the last pelleted fraction, Fraction E (21 000 × g, 60 min), only contained 3 – 5 % of the total protein. Based on the organelle density, proteins from e.g., plasma membrane and endoplasmic reticulum organelles were expected to be collected in Fraction E. However, these proteins were found in all fractions, with on average 60% in Fraction A (Figure 8a). This is in agreement with previous findings that the majority of proteins are collected already in the first, low-speed centrifugation step, and can explain the loss of proteins in membrane fractions obtained by subsequent high-speed centrifugation.[27]

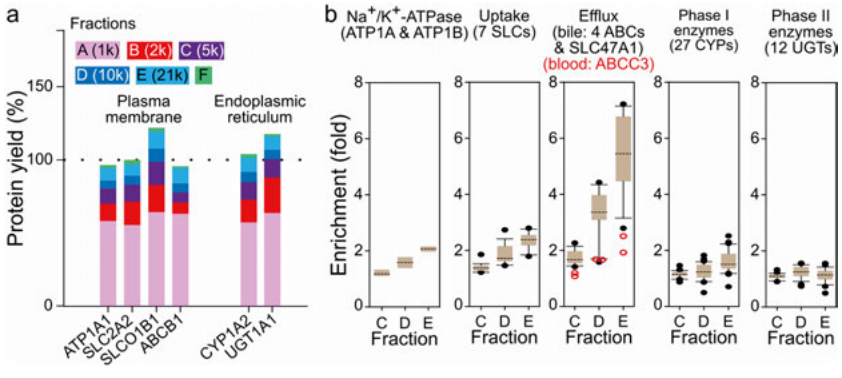

Figure 8. Distribution of proteins relevant for drug disposition in different fractions obtained after differential centrifugation. (**a**) Protein yield (%) and partition of specific membrane proteins in the subcellular fractions relative to the homogenate. (**b**) Protein enrichment values of membrane proteins in fractions C, D, and E as compared to concentrations in fraction B.

Drug transporting proteins, located at the plasma membrane, were also spread out over the different fractions, but were found in continuously higher amounts in the fractions obtained by higher centrifugal speed (Fractions C, D and E; Figure 3b). Interestingly, transporters located at the basolateral and canalicular membrane displayed different enrichment profiles across the fractions. Uptake and efflux transporters at the basolateral membrane were distributed with similar profiles across the three fractions, whereas efflux transporters at the canalicular membrane were found in much higher concentrations in Fraction E (Figure 8b).

Drug metabolizing CYP and UGT enzymes located in the endoplasmic reticulum were more evenly spread across the fractions, with less enrichment in Fraction E as compared with the transporters. The variability in enrichment profiles indicates a complex distribution of membrane proteins over fractions obtained from differential centrifugation and can explain the low enrichment of proteins from the laboratories with subcellular fractionation processes in Paper I. The enrichment variability also limits the usefulness of using single membrane markers, such as ATP1A1 (Na^+/K^+-ATPase) to account for the enrichment of membrane proteins.[173,174]

Impact of differences in protein quantification on pharmacokinetic predictions

Protein quantification is often used in predictive pharmacokinetics for modeling of *in vitro–in vivo* correlations. Thus, in Paper I, we also assessed the impact of variable protein quantification on predictions of intrinsic liver clearance, from the six laboratories. For this purpose, we used a previously published static model for estimating the maximal uptake clearance of atorvastatin based on protein levels of the uptake transporters SLCO1B1, SLCO1B3, and SLCO2B1[60], quantified with the different methodologies (Figure 9a). The overall predicted uptake clearance varied on average seven-fold across the laboratories when using protein levels quantified in whole tissue digests and enriched proteins scaled to whole tissue (Figure 9b). Together, this demonstrates that differences in protein quantification from different methodologies strongly influence predictions of liver clearance.

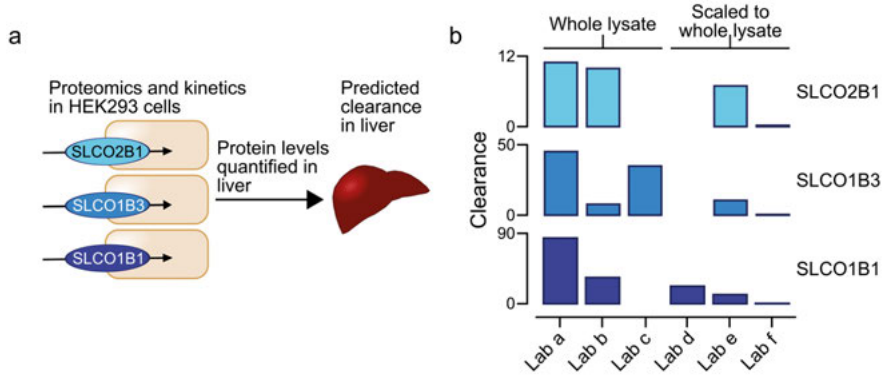

Figure 9. Effect of variable levels of transporter proteins on clearance predictions of atorvastatin. (**a**) Schematics of the prediction model.[60] (**b**) Predicted uptake clearance of atorvastatin by SLCO1B1, SLCO1B3, and SLCO2B1 based on the protein quantities obtained in Labs a–c (fmol/µg total protein) and Labs d–f (protein levels scaled to whole lysate; fmol/µg total protein). The contribution of each transporter is represented by geometric mean values of calculated clearance from the ten liver samples. Labs c and d only quantified SLCO1B3 and SLCO1B1, respectively.

Effects of protein content and intracellular unbound drug concentration on metabolic activity (Paper III)

Human liver microsomes (subcellular fraction obtained after ultracentrifugation at 104 000 × g, 60 min) are commonly used to study drug metabolism *in vitro*, due to the low cost and ease of access compared to the gold standard hepatocytes. In Paper III, proteomics and intracellular unbound drug concentration analysis were used to investigate differences in intrinsic drug clearance of specific CYP probes: bupropion (CYP2B6), bufuralol (CYP2D6), diclofenac (CYP2C9), omeprazole (CYP2C19), and midazolam (CYP3A4), obtained in human liver microsomes and donor-matched isolated hepatocytes.

Global proteomics analysis of human liver homogenates, hepatocytes, and liver microsomes

Global proteomics analysis was performed on human liver homogenates, hepatocytes, and liver microsomes from 15 matched donors. Principal component analysis (PCA) of the proteomics data showed that the microsomal samples differed from both other sample types along the first principal component axis, which describes most of the data variability (PC1 46.2%; Figure 10a). It has been suggested since a long time that microsomes are vesicles derived from the ER.[120] In this study, proteins with significantly higher concentrations in the microsomes compared to in both homogenates and hepatocytes were involved in pathways associated with the ER. The proteins were

involved in processes such as cholesterol and sphingolipid synthesis, vesicle transport from the ER, fatty acid metabolism, and drug metabolism. Proteins annotated to the ER also made up a larger proportion of the total protein content in the microsomal samples (19%) than those in the homogenates (7%) and hepatocytes (9%) (Figure 10b). However, the microsomes also contained proteins annotated to other subcellular compartments such as the mitochondria (14% of the total microsomal protein content) and nucleus (19%). Microsomes also contained similar proportions of cytosolic proteins as both homogenates and hepatocytes (27%; Figure 10b).

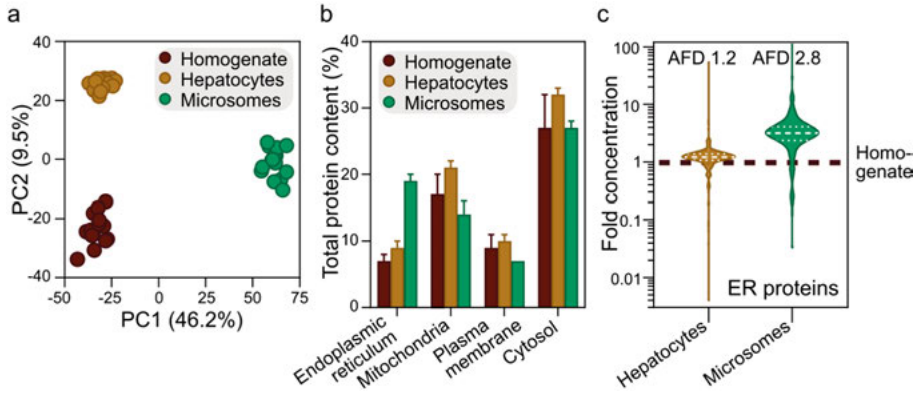

Figure 10. Global proteomics analysis of human liver homogenates, isolated hepatocytes, and liver microsomes. (**a**) Principal component analysis of proteins quantified with at least three peptides in liver homogenates, hepatocytes, and microsomes, obtained from 15 donors. (**b**) Proportion of total protein content in different subcellular locations (proteins annotated by HPA)[157]. Bars show average levels and error bars denote standard deviation from the 15 donors. (**c**) Fold concentration of ER-located proteins in hepatocytes and microsomes, respectively, compared to liver homogenates. AFD denote average fold differences.

ER-annotated proteins were also generally enriched to higher concentrations in the microsomal samples than in the homogenates (Figure 10c), but only with an average enrichment of 2.8-fold. Since the ER-related proteins constituted 7% of the total protein content in the liver homogenates, a 14-fold enrichment would be expected in the microsomal samples, assuming complete isolation and full recovery of the ER fraction in the microsomes. The lower enrichment in microsomes is probably an effect of loss of proteins in the fractionation process,[27] as was observed in Paper II, in combination with the contamination of proteins from other subcellular compartments.

CYP enzymes in human liver homogenates, hepatocytes, and microsomes

The isolation of ER-localized proteins in liver microsomes should lead to enhanced levels of membrane-bound drug metabolizing enzymes, such as CYPs. In general, CYP enzymes were enriched 3.2-fold in the microsomes compared to in both liver homogenates and hepatocytes (Figure 11), similar to what was observed for enzymes in enriched subcellular fractions in Paper I. Interestingly, two of the enzymes from the subfamily CYP2C (CYP2C9 and CYP2C19) were very differently enriched in the microsomes. CYP2C9 had higher homogenate concentrations but showed poor enrichment (average 1.4), while CYP2C19 had lower homogenate concentrations but was enriched 50-fold in the microsomes. In line with this, global analysis of the microsomal protein enrichment compared to the initial protein concentration in the homogenate, showed that proteins with higher concentration tended to be less enriched in the microsomes compared to those with lower concentrations ($r_s = -0.33$), suggesting a saturation in the enrichment process. Further, the variability in protein levels between donors of the different CYP enzymes was generally in agreement between the liver homogenates (median inter-donor variability across CYPs: 3), hepatocytes (median: 7), and microsomes (median: 3). In contrast, the inter-donor variability of the highly enriched CYP2C19 was only three-fold in microsomes, compared to 86-fold in the homogenates.

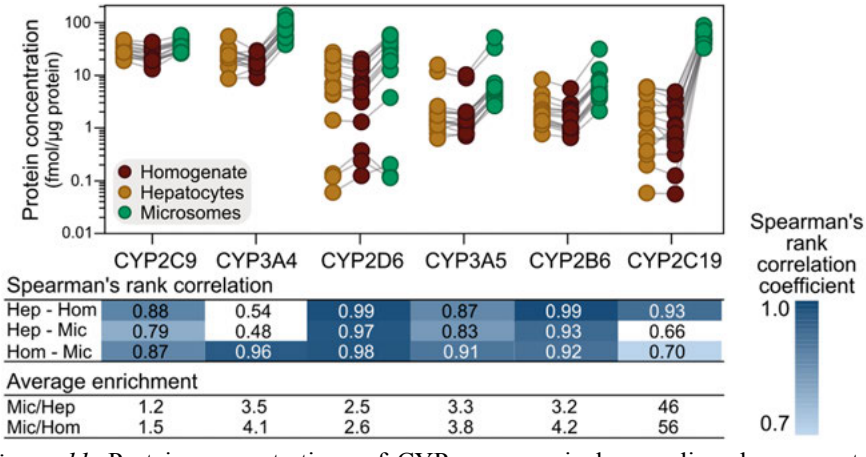

Figure 11. Protein concentrations of CYP enzymes in human liver homogenates, hepatocytes, and microsomes from the 15 donors. Concentration levels are given in fmol/µg total protein in the respective system. Spearman's rank correlations compare the relative expression of each enzyme across the 15 donors between the respective sample types, in which significant correlation coefficients are > 0.7 ($P < 0.006$, after Bonferroni correction for multiple comparisons). Average enrichment of microsomes compared to homogenate and hepatocytes were calculated based on protein concentrations from the 15 donors.

The donor rank order based on expression levels for the different CYPs were generally highly correlated between the different sample types, with median Spearman's correlation (r_s) of 0.90 between homogenates and microsomes, 0.76 between hepatocytes and microsomes, and 0.87 between hepatocytes and homogenates (Figure 6). However, CYP3A4 and CYP2C19 displayed weaker correlations between microsomes and hepatocytes from the 15 donors (r_s = 0.48 and 0.66, respectively). Since these two enzymes are important for drug metabolism, the differences in protein expression in the two commonly used *in vitro* systems could have further implications on metabolic activity assays.

Intracellular unbound drug concentrations (Kp_{uu}) of CYP probes in human hepatocytes

To further investigate factors that could influence the intrinsic metabolic clearance of CYP probes in hepatocytes and microsomes, intracellular unbound drug concentrations were determined in human hepatocytes. For some drugs, passing the intact plasma membrane barrier in the hepatocytes can be the rate-limiting step for their clearance. Since the barrier is not present in the microsomes, this factor could give rise to higher intrinsic clearance as compared to in the hepatocytes. Intracellular unbound concentrations (as measured by Kp_{uu}) of the CYP probes varied among the different compounds (Figure 12). Omeprazole, bupropion, and diclofenac had Kp_{uu}-values close to one, with geometric mean values of 1.4, 1.2, and 0.71, respectively, across the 15 donors. This indicates that the unbound concentrations were similar on the outside and inside of the hepatocytes. Midazolam had lower Kp_{uu}-values (mean Kp_{uu} = 0.32), showing that the drug distribution was shifted towards the outside of the hepatocytes during the experiment, possibly due to incomplete equilibrium of the rapidly metabolized compound, and active efflux transport.[175] In contrast, bufuralol had higher Kp_{uu}-values (mean Kp_{uu} = 2.5) suggesting an accumulation of the drug inside of the hepatocytes (Figure 12). Since bufuralol is a basic compound, it can be trapped in subcellular compartments with low pH, such as the lysosome[176]. Accordingly, we found that the mean Kp_{uu}-values of bufuralol in hepatocytes was reduced to 1.2 when bufuralol was co-incubated with the lysosomotropic agent bafilomycin A1, a known inhibitor of the lysosomal proton pump V-ATPase, which reduces the lysosomal pH gradient.[177,178]

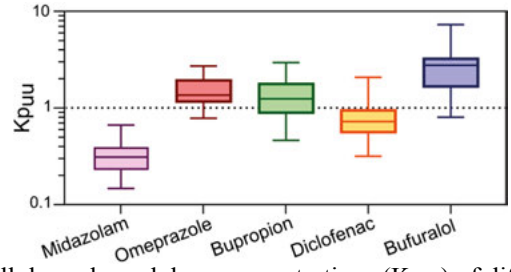

Figure 12. Intracellular unbound drug concentrations (Kp_{uu}) of different CYP probes in human hepatocytes from 15 donors. The boxes denote the 25th and 75th percentiles, The line denotes the median value, and whiskers denote minimum and maximum values from the 15 donors.

Explaining the differences in microsomal and hepatocyte clearance activities by protein content and Kp_{uu}

The intrinsic clearance of midazolam (probe substrate for CYP3A4) was higher in the microsomes (geometric mean unbound $CL_{int,mic}$ = 250 µl/min/mg protein) than in the hepatocytes ($CL_{int,hep}$ = 74 µl/min/mg protein; Figure 13a). To investigate if the higher intrinsic microsomal clearance could be partly explained by cellular accumulation of the unbound drug in hepatocytes, and by the content of CYP enzyme in the hepatocytes and microsomes, these parameters were used to adjust the $CL_{int,mic}$. When Kp_{uu}-values from the 15 donors were used to adjust the respective microsomal clearance values, the $CL_{int,mic}$-values were lowered closer to the $CL_{int,hep}$-values (Figure 13b). The average fold difference (AFD) between $CL_{int,hep}$ and the adjusted $CL_{int,mic}$ ($CL_{int,corr}$) decreased from 5.6 to 1.8.

Further, the poor rank–order correlation of CYP3A4 levels in microsomes and hepatocytes (Figure 11) was reflected in poor donor rank–order correlation of clearance values from $CL_{int,mic}$ and $CL_{int,hep}$ (r_s = 0.55). Adjustment of $CL_{int,mic}$ with the CYP3A4 content in hepatocytes and microsomes improved the rank–order correlations (r_s = 0.79) of the clearance values in the two *in vitro* systems (Figure 13c). Together, these results showed that Kp_{uu} and CYP3A4 content could explain the higher clearance of midazolam in microsomes and the poor ranking of donors, which was projected in decreased AFD and increased correlation between two *in vitro* systems (Figure 13d).

Kp_{uu} and CYP content also explained the higher microsomal clearance of omeprazole (probe substrate for CYP2C19), where adjustment of $CL_{int,mic}$ decreased the AFD from CLint,hep from 3.8 to 0.6 and the rank order increased with r_s-values from 0.90 to 0.96. However, the Kp_{uu} and CYP content alone could not explain the higher microsomal metabolism rate of diclofenac that has a more complex metabolism pathway with the involvement of several phase I and phase II metabolizing enzymes only active in hepatocytes.[179] In contrast, the clearance of bupropion and bufuralol were comparable in microsomes and hepatocytes.

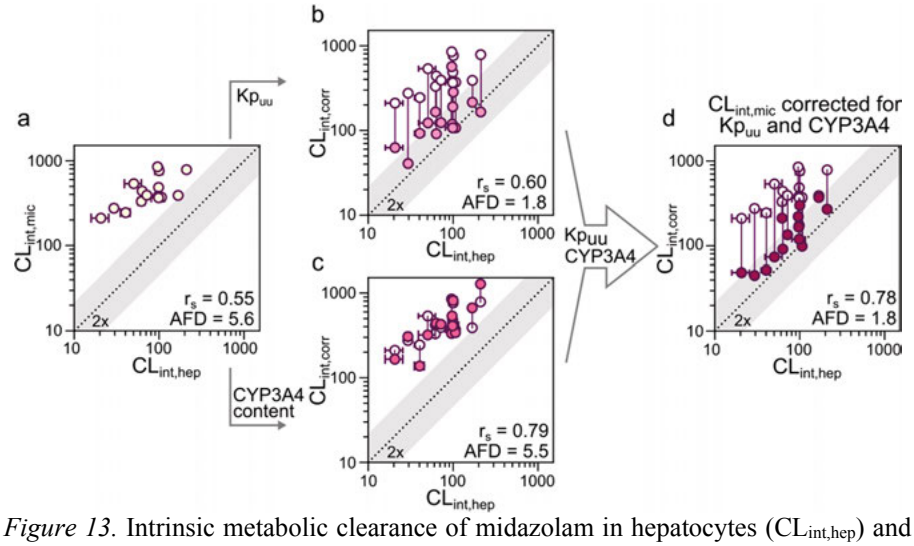

Figure 13. Intrinsic metabolic clearance of midazolam in hepatocytes ($CL_{int,hep}$) and microsomes ($CL_{int,mic}$). $CL_{int,mic}$ was corrected with intracellular unbound drug concentration (Kp_{uu}) from hepatocytes, CYP3A4 content in hepatocytes and microsomes, or a combination of Kp_{uu} and CYP3A4 content ($CL_{int,corr}$). $CL_{int,mic}$ and $CL_{int,hep}$ were measured in µl/min/mg total protein in the incubations. AFD denotes the average fold difference and r_s denotes the Spearman's rank correlation coefficient.

Protein expression variability analysis within human liver and jejunum from different donors (Paper IV)

In-depth knowledge of inter-individual expression variability in single tissue types would enable better representations of entire populations in systems biology, which would advance the field of personalized medicine.[180,181] Inter-individual differences in tissues have been investigated extensively at the mRNA level,[182,183] but this has so far not been comprehensively investigated at the protein level. Therefore, in Paper IV, inter-individual variability in protein expression in human liver and jejunum was studied in biopsies obtained from 38 donors undergoing gastric bypass surgery. To simplify the variability analysis, Gini indices were calculated from protein levels obtained by global proteomics from the 38 donors for both the human liver and jejunum. The Gini index is a measure of variability in a dataset,[184] and was recently introduced for describing gene expression variability across different tissue types.[72] The Gini index ranges from 0 to 1, with lower values indicating similar expression levels across samples.

Variability in protein concentrations in single tissue types

In general, the inter-individual expression variability of jejunal and liver proteins were lower than the expression variability observed across 29 different

human tissues (Figure 9a).[185] Furthermore, the variability distributions of all proteins quantified in the jejunal samples was shifted towards higher values compared to liver – the Gini indices ranged between 0.04–0.94 in jejunum and between 0.01–0.84 in liver ($P < 0.0001$, one-way ANOVA followed by Tukey's multiple comparisons test; Figure 9a). This demonstrates that large inter-individual differences are obtained for many of the proteins in both tissue types. The overall higher variability in jejunum could be explained by the liver being a relatively homogenous tissue, mainly constituted by hepatocytes (80% of the liver volume),[186] whereas the jejunum is composed of several distinct tissue layers.[187] The layered nature of the jejunum means that the pinch-biopsy technique used for obtaining the jejunal samples can result in slightly different sample compositions due to sampling depth,[188] where higher proportions of collagen-rich submucosal connective tissue could dilute the tissue-specific expression of the jejunal epithelium to varying degrees. Further, inter-individual differences in jejunum length[189] could affect the relative position of the jejunal sampling site, and thus influence the variability due to regional differences in protein expression in the human small intestine.[79]

Gini indices of each protein were correlated in liver and jejunum ($r = 0.48$; 5496 proteins; Figure 14b). This demonstrates that the overall variability in proteins across the donors is relatively similar in liver and jejunum.

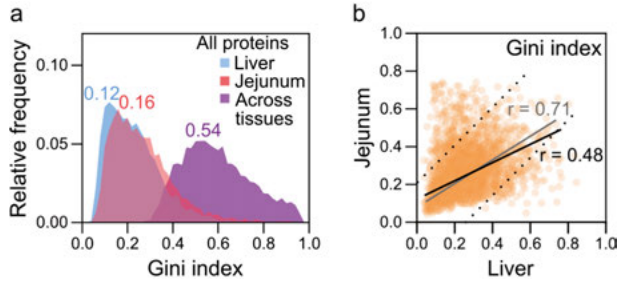

Figure 14. Variability in protein expression in single tissue types. (**a**) Variability distribution of proteins in human liver and jejunum from 38 donors, and in proteomics data across 29 tissues[185], comparing within-tissue and across-tissue variability. Numbers in figure denote the mode. (**b**) Correlation of protein expression variability between the liver and jejunum datasets. The black line shows the regression with all proteins included, and the grey line shows the regression after exclusion of highly discrepant proteins (outside dotted lines) between the two tissues and r denotes Pearson's correlation coefficient.

Biological processes among proteins with low and high expression variability

Next, an in-depth analysis of the within-tissue variability in the proteomes of the 38 liver and jejunum samples was performed. The 100 least variable pro-

teins represented similar basal cellular functions in both tissues, such as carbohydrate metabolism, protein processing and translation. Also, proteins selected from the 'core essentialome',[190] i.e., proteins that are essential for cell survival, had significantly lower Gini indices and thus similar protein expression across the donors in both liver and jejunum ($P < 0.0001$, Student's t-test; Figure 15a). Cells need to maintain these proteins at certain levels to ensure survival and therefore they had low Gini indices in both tissue types.

In contrast, disease-related proteins were among the proteins with high expression variability across donors. For instance, proteins annotated with 'inflammatory response' by Gene Ontology,[191] and proteins associated with diabetes in the DisGeNET database[192] had significantly higher Gini indices compared to proteins from core cellular structures, such as the proteasome and ribosome with expected low expression variability, in both liver and jejunum (one-way ANOVA followed by Dunnett's multiple comparisons test). Furthermore, proteins important for drug metabolism, i.e., phase I and phase II metabolizing enzymes, covered large parts of the variability spectrum in both liver and jejunum (Figure 15b). Gini indices of CYPs responsible for the majority of phase I drug metabolism[78] ranged from 0.09–0.34 and 0.19–0.88 in liver and jejunum, respectively, while Gini indices for the most important phase II metabolizing UGTs[77] ranged from 0.11–0.46 in liver and 0.17–0.88 in jejunum. The high inter-donor variability in expression levels of drug metabolizing enzymes implies that individual expression levels, as opposed to population averages, should be considered for tailored systems biology analysis.

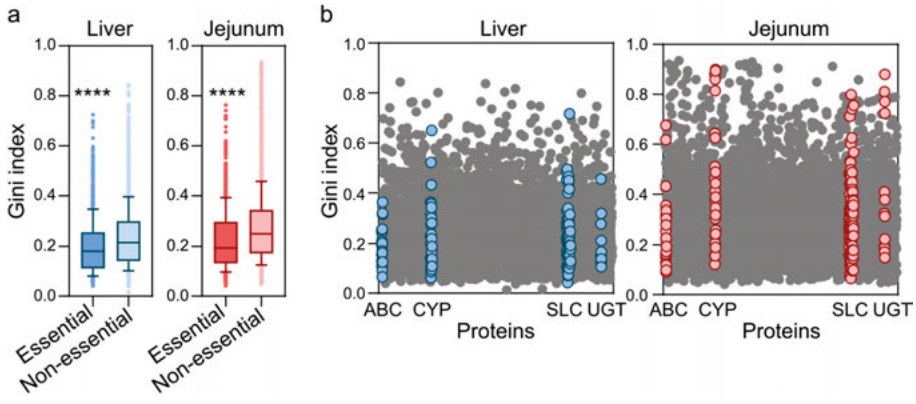

Figure 15. Biological processes represented by proteins with low and high variability, respectively, in single tissue types. (**a**) Gini indices of essential and non-essential proteins in human liver and jejunum from 38 donors. Boxes range between the 25th and 75th percentiles, lines show medians, and whiskers denote 10th and 90th percentile. **** $P < 0.0001$, one-way ANOVA followed by Dunnett's multiple comparisons test. (**b**) Gini indices of all proteins quantified in human liver and jejunum ordered by gene name. Important transporter and metabolic enzyme families are highlighted.

Effects of obesity, gastric bypass surgery, and weight loss on global protein expression in human liver and jejunum (Paper V)

The global proteomics data analysis of human liver and jejunum biopsies obtained from patients with obesity was extended to investigate how obesity, gastric bypass surgery, in which part of the small intestine is removed and the jejunum is attached directly to the gastric pouch, and weight loss affect protein expression in the two tissue types. For this purpose, the proteomes of jejunal biopsies obtained from matching donors with obesity at the time of gastric bypass surgery (n = 37), and biopsies through endoscopy six weeks later (n = 34), and two years after surgery (n = 11) were analyzed. Prior to the surgery, the patients underwent a three week period on a low calorie diet with less than 1200 kcal per day.[193] At the different time points, several clinical parameters were collected from the patients to monitor e.g., blood pressure, electrolyte-, glucose-, fat-, and inflammation levels. During the study period, the patients reduced their median weight from 129 kg (median BMI 45 kg/m^2) three weeks prior to surgery (–W3), to 128 kg (BMI 43 kg/m^2) at the time of surgery (W0), 113 kg (BMI 40 kg/m^2) six weeks after surgery (W6), and 96 kg (BMI 30 kg/m^2) two years later (Y2; Figure 16a).

At the time of gastric bypass surgery, liver biopsies were also collected from the patients with obesity (Ob, n = 38). It was not possible to collect liver biopsies through endoscopy six weeks and two years after the study. Therefore, the effect of weight loss on the liver proteins in the same patients could not be studied. Instead, the liver proteomes of the patients with obesity were compared with those obtained from non-obese patients (n = 17; median weight and BMI of 73 kg and 24 kg/m^2, respectively) undergoing cholecystectomy (gallbladder removal; Figure 17a).

Protein profiling of human jejunum from patients with obesity undergoing weight loss from gastric bypass surgery

Principal component analysis of the global proteomics data obtained from the jejunal samples at the three time points showed that biopsies taken six weeks (W6) and two years (Y2) after the surgery differed from the samples taken at the time of surgery (W0) along the most influential first principal component axis (Figure 16b). Although only a small proportion of the total variance in the proteomics dataset was explained by the first component (PC1 11%), *limma* analysis provided statistically different expression levels of proteins across the groups ($P < 0.05$, expression fold-change > 1.5, adjusted with Benjamini-Hochberg). Proteins that were higher at W0 compared to at W6 and Y2 were involved in processes such as fatty acid β-oxidation, and triglyceride biosynthesis and transport. This was likely an artifact of the three weeks of low

calorie intake prior to surgery, since fasting activates the degradation of stored fatty acids through mitochondrial fatty acid β-oxidation in the cells.[194]

Further, lower levels of proteins involved in immune response, such as T-cell receptor signaling, were found at Y2 compared to at W0 and W6. This was in agreement with decreased systemic inflammation markers measured in the same patients one year post-surgery. Obesity is associated with increased inflammatory processes and immune response,[89,195] and the data indicate that the jejunal inflammatory response decreases as an effect of weight loss in the patients two years post-surgery.

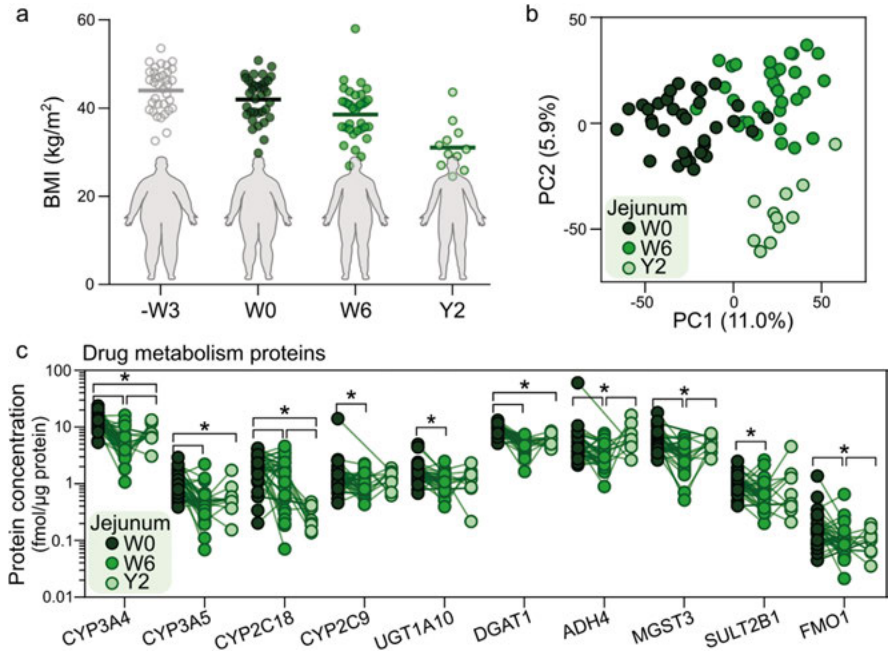

Figure 16. Proteomics analysis of jejunal biopsies. (**a**) Schematic representation of different time points for visits (–W3, W0, W6, and Y2), with corresponding BMI values of the patients. (**b**) Principal component analysis of jejunal proteins quantified in biopsies taken at W0, W6, and Y2. (**c**) Concentrations of jejunal proteins involved in drug metabolism, with significantly lower values at W6 compared to W0. –W3, three weeks fasting period prior to gastric bypass surgery; W0, jejunal biopsies at time of surgery; W6, jejunal biopsies six weeks after surgery; Y2, jejunal biopsies two years after surgery.

The differential expression analysis also showed that proteins involved in drug metabolism were decreased at W6 compared to W0 in the jejunum. These proteins included phase I drug metabolizing CYP enzymes and one phase II drug metabolizing enzyme, and were on average 1.8-fold lower in the W6 samples ($P < 0.05$), with half of the proteins remaining at these levels at Y2 (Figure 16c). This suggests that drugs could be metabolized more rapidly in the jejunum with a potential reduction of the bioavailability in patients with

obesity prior to gastric bypass surgery. Whether this is an effect of the gastric bypass surgery or an effect of weight loss needs to be explored further.

Protein profiling of human liver from patients with and without obesity

No clear differences were observed in the liver proteomes of the patients with obesity (Ob) undergoing gastric bypass surgery and the non-obese (NonOb) patients undergoing cholecystectomy from the principal component analysis (Figure 17b). Similarly, *limma* analysis of the two patient groups provided only a few significantly different proteins. Among these, several perilipins involved in lipid storage and inflammation were expressed at higher levels in the Ob patients, confirming that lipid storage is upregulated in patients with obesity. In contrast, collagen proteins important in the formation of extracellular matrix had higher expression levels in the NonOb patients, suggesting that the livers were more fibrotic.

There were no significant differences in liver proteins involved in drug disposition between the patients with and without obesity. However, trends were observed between liver proteins involved in drug disposition and body weight. For instance, the efflux transporter ABCB1 had a positive relationship with body weight ($r_s = 0.40$), while both of the phase I and phase II drug metabolizing enzymes CYP3A4 and UGT1A3 had negative relationships with body weight ($r_s = –0.34$ and $r_s = –0.36$, respectively). CYP3A4 has previously been shown to correlate negatively with body weight, but with stronger correlation than what was observed here.[85] These varying protein levels suggest that drug disposition can vary in patients as an effect of body weight.

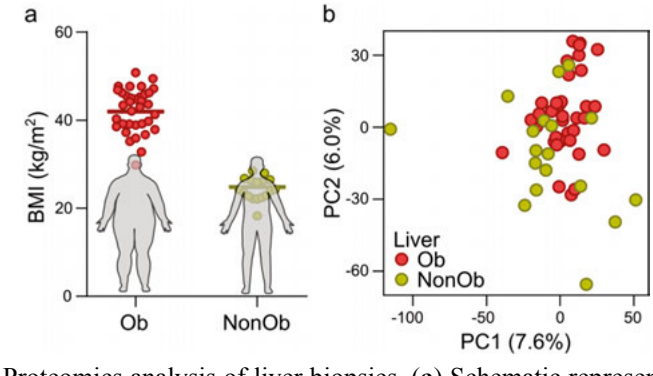

Figure 17. Proteomics analysis of liver biopsies. (**a**) Schematic representation of the liver sample types, with corresponding BMI values from the included patients. (**b**) Principal component analysis of liver proteins quantified in the Ob and NonOb biopsies. Ob, liver biopsies from patients with obesity at gastric bypass surgery; NonOb, liver biopsies from non-obese patients at cholecystectomy.

Comparison of protein concentrations and clinical parameters

Next, the relationship between protein concentrations and corresponding clinical parameter values obtained from all patients was investigated. Several interesting correlations were found in the jejunum. For instance, the concentration of the ribosomal protein RPS4Y1, involved in fatty acid metabolism,[196] increased with increased fat free body mass and muscle mass (Figure 18a; r_s = 0.63 and 0.58, $P < 0.05$, adjusted with Benjamini-Hochberg for multiple comparisons, respectively).

In the liver, several liver proteins associated with insulin regulation and signaling correlated with insulin levels. For instance, the two perilipins PLIN1[197] and PLIN2[198] were both positively correlated with insulin (Figure 18b; r_s = 0.64, $P < 0.05$, adjusted with Benjamini-Hochberg and r_s = 0.59). Thus, these clinical markers and proteins could be suitable components for biomarkers or targets for inducing weight loss.

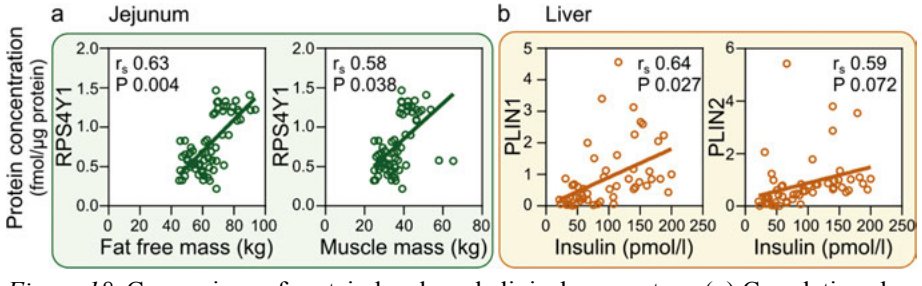

Figure 18. Comparison of protein levels and clinical parameters. (**a**) Correlations between jejunal protein concentrations and clinical parameters. (**b**) Correlations between liver protein concentrations and clinical parameters, where r_s, is the Spearman's rank correlation (significant correlations when $P < 0.05$, adjusted with Benjamini Hochberg).

Proteomics-informed prediction of rosuvastatin clearance in donors with and without obesity (Paper VI)

In the final study (Paper VI), the liver proteomics data obtained from the patients with obesity and non-obese patients was used to create a proteomics-informed mechanistic model to predict the plasma pharmacokinetics of rosuvastatin. The model was based on previously established models in which uptake activity from cell lines expressing single transporters are scaled to the *in vivo* situation using protein levels in the cell model and *in vivo*, with the assumption that the maximal transport activity depends on the transporter expression (eq. 9).[48,49,60,160] Although rosuvastatin has been widely used in pharmacokinetic investigations and modeling of transporter-mediated uptake and

drug-drug interactions,[48,49,149,163,199] these studies did not consider the contribution of inter-individual transporter expression. Further, unrelated *in vivo* data has been used to evaluate the predictions.

In this study, *in vitro* uptake kinetics from HEK293-transfected cells were combined with protein quantification to assess the contribution of SLCO1B1, SLCO1B3, SLCO2B1, and SLC10A1 (the major hepatic uptake transporters of rosuvastatin[35,48,49]) to the rosuvastatin clearance in 56 donors with varying hepatic transporter expression. These parameters were used in a bottom-up approach to build a mechanistic model to predict the plasma pharmacokinetics of rosuvastatin in the 56 donors. The results from the prediction model was then compared with plasma concentration profiles of rosuvastatin in the 56 donors, collected after an oral dose of 20 mg rosuvastatin the day before surgery.

Establishing *in vitro* parameters for the prediction of rosuvastatin pharmacokinetics

The transporter-mediated uptake of rosuvastatin in the HEK293 cells was used to determine the pharmacokinetic parameters V_{max} and K_m for the four hepatic uptake transporters (Figure 19a). The measured pharmacokinetic parameters were in line with that described previously.[35,48] The concentration of each transporter in the respective cell system was determined with targeted mass spectrometry-based proteomics and ranged between 0.9 to 6.2 pmol/mg protein (Figure 19a).

The four uptake transporters were quantified with global proteomics in the 56 liver biopsies undergoing two types of surgery: gastric bypass (Ob, 38) and cholecystectomy (NonOb, 18). There were no statistical differences in the protein levels of either of the transporters between the two patients groups. However, large inter-individual differences in the levels were observed for all transporters, in which SLCO1B1 ranged from 0.9 to 5.5, SLCO1B3 from 0.3 to 2.6, SLCO2B1 from 0.4 to 1.2, and SLC10A1 from 0.04 to 2.5 pmol/mg protein across the Ob and NonOb patients (Figure 19b). These large differences in protein levels could thus affect the rosuvastatin uptake across the donors.

a

Transporter	HEK293 K_m	HEK293 V_{max}	HEK293 Protein concentration	Ob Protein concentration	NonOb Protein concentration
	µM	pmol/min/mg protein	pmol/mg protein	pmol/mg protein	pmol/mg protein
SLCO1B1	**9.5** ± 1.9	**24.5** ± 1.9	**1.33**	**2.63** (1.13 - 5.46)	**2.37** (0.88 - 3.93)
SLCO1B3	**17.2** ± 2.9	**22.1** ± 1.8	**1.11**	**1.03** (0.45 - 1.90)	**1.40** (0.28 - 2.58)
SLCO2B1	**14.7** ± 2.2	**6.8** ± 0.4	**6.2**	**0.83** (0.42 - 1.22)	**0.80** (0.50 - 1.10)
SLC10A1	**192** ± 25.6	**300** ± 18.3	**0.9**	**0.31** (0.04 - 0.72)	**0.37** (0.12 - 2.51)

b

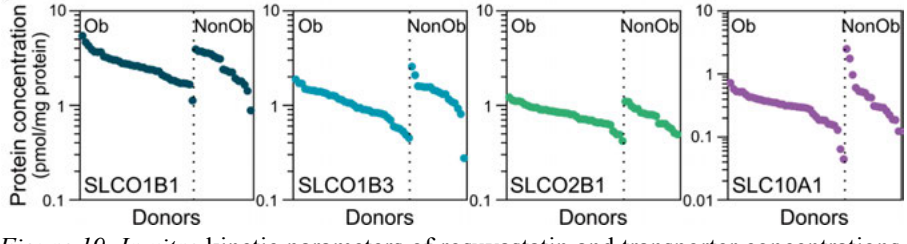

Figure 19. In vitro kinetic parameters of rosuvastatin and transporter concentrations. (**a**) Kinetic parameters (K_m and V_{max}) of rosuvastatin in HEK293 cells overexpressing SLCO1B1, SLCO1B3, SLCO2B1, or SLC10A1 (values represent mean values with standard deviation obtained from duplicates analyzed on at least two different occasions) and protein concentrations of the respective transporters in the overexpressing cells and liver biopsies (Ob and NonOb, protein levels expressed as median with range across the donors). (**b**) Protein concentration levels of the four uptake transporters in the 56 liver biopsies (Ob and NonOb). Ob, patients with obesity from gastric bypass surgery; NonOb, non-obese patients from cholecystectomy.

Concentration–time profile of rosuvastatin clearance *in vivo*

The time-dependent distribution of rosuvastatin demonstrated a rapidly appearing initial peak in plasma concentration (median C_{max} 7.4 ng/ml in Ob, and 9.2 ng/ml in NonOb), and a slower, biphasic elimination for most of the patients (Figures 20a and 20b). There was no statistical difference in the pharmacokinetic parameters of rosuvastatin distribution between the Ob and NonOb patients. However, large inter-individual differences was observed in the time-dependent disposition of rosuvastatin. Across the 56 patients, the C_{max} varied from 1.9 to 56.6 ng/ml, the area under the curve ($AUC_{0\text{-}24h}$) ranged from 18.3 to 237.1 ng/ml/h, and the terminal half-life ($T_{1/2}$) ranged from 4.1 to 50.4 h (Figures 20a and 20b). Although the pharmacokinetic parameters varied more across the Ob donors that had large spread in weight (79.2 – 175.8 kg as compared to 47.4 – 96.5 kg in NonOb), there was no correlation between any of the parameters and body weight, suggesting that body size is not the determining factor of rosuvastatin disposition.

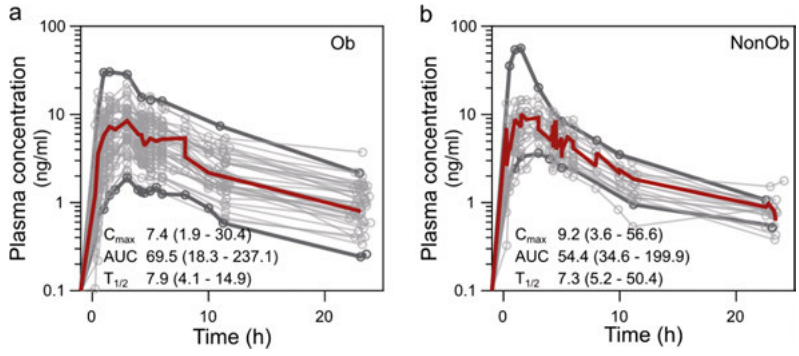

Figure 20. Time-dependent disposition of rosuvastatin in plasma in patients undergoing (**a**) gastric bypass (Ob) and (**b**) cholecystectomy (NonOb). Red thick lines represent the mean disposition of all donors undergoing the respective surgery. Thick grey lines represent disposition with lowest and highest area under the curve (AUC). Ob, patients with obesity from gastric bypass surgery; NonOb, non-obese patients from cholecystectomy; C_{max} (ng/ml); AUC (ng/ml/h); $T_{1/2}$ (h).

Prediction of time-dependent disposition of rosuvastatin in plasma

With a simple mechanistic model (using eq. 10) to predict time-dependent disposition of rosuvastatin in the 56 donors, 60% of the predicted AUC values were within two-fold error from the observed values. The average AFE of the AUC values was 0.64, indicating a bias towards under-prediction of the observed AUC. The model was better at predicting the C_{max}, with the AFE being 0.89, and 74% of the predictions being less than two-fold from the observed values.

In contrast, the model consistently under-predicted the $T_{1/2}$, giving AFE of 0.38 between predicted and observed values across the 56 donors. It was also clear that this model did not capture the biphasic elimination observed for rosuvastatin. Local sensitivity analysis of all the parameters in the model demonstrated that these did not affect the biphasic elimination behavior.

Enterohepatic recirculation of rosuvastatin is important for its plasma distribution[48,162] and this was considered in the model by adding an additional compartment representing the gallbladder. With this additional compartment (eq. 11; Figure 21a), the predictions of all three pharmacokinetic parameters, AUC, C_{max}, and $T_{1/2}$ improved (AFE between predicted and observed = 0.95, 0.91, and 0.69). Although the predicted parameters were improved, the model still did not fully capture the biphasic elimination behavior observed for rosuvastatin, using a previously established emptying rate from the gallbladder to the intestine.[167] The gallbladder emptying rate can decrease with body size and gallbladder volume,[167] and when the emptying rate in the model was lowered, the model could capture the biphasic elimination behavior (Figures 21b and

c). The need to optimize the emptying rate in this model could also be explained by the complexity in the delay and rate of gallbladder emptying, in which the delay and rate is strongly influenced by meal intake,[200] which was not controlled in this study.

With this optimization of the final model with a gallbladder compartment, $T_{1/2}$ was also predicted within two-fold error, with good precision and no bias towards under-or over-prediction (AFE of 0.96), similar to the predicted AUC and C_{max} (Figures 21b and 21c).

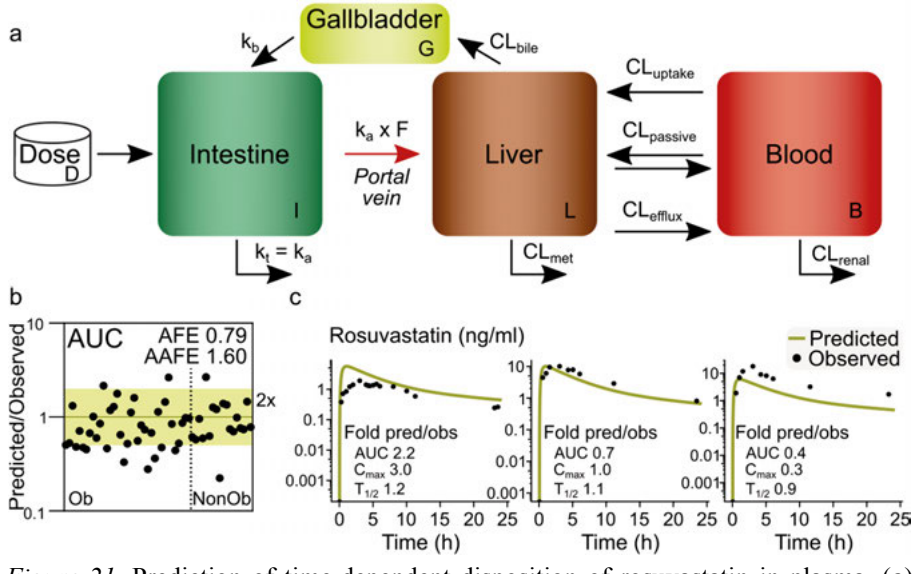

Figure 21. Prediction of time-dependent disposition of rosuvastatin in plasma. (**a**) Schematics of mechanistic model with gallbladder compartment. (**b**) Fold values from predicted and observed AUC of rosuvastatin from the 56 donors. (**c**) Representative examples of donors with low, medium, and high fold value between predicted and observed AUC. Ob, patients with obesity from gastric bypass surgery; NonOb, non-obese patients from cholecystectomy; C_{max} (ng/ml); AUC (ng/ml/h); $T_{1/2}$ (h).

Influence of genetic variants on the prediction outcome

Among the 56 donors, one third (18) had the genetic variant SLCO1B1*5 that is associated with reduced hepatic uptake activity of statins.[201] The lower uptake activity was observed as patients with the SLCO1B1*5 variant had increased C_{max} (159%) and AUC (163%) values compared to those with the reference SLCO1B1*1a variant ($P < 0.05$, Mann-Whitney test; Figure 22a). This was also reflected in significant under-prediction of both C_{max} and AUC for patients with the SLCO1B1*5 variant ($P < 0.05$, Kruskal-Wallis test), since the predicted plasma concentrations of rosuvastatin was lower than that observed for these patients (Figure 22b). SLCO1B1*5 has been shown to have approximately 10% uptake activity compared to the *1a variant.[35] When this

reduced activity was adjusted for in the model, the predictions of C_{max} and AUC for the patients with the *5 variant improved to the levels of patients with the *1a variant (Figure 22b). It has been suggested that the reduced activity of the *5 variant is caused by interference of the allele with the localization of the transporter to the plasma membrane.[201] The under-prediction could thus be an effect of the protein quantification. The proteomics method used to quantify the proteins in this study does not discriminate for transporters at the membrane but measures all transporters in the cell, which could give an overestimation of the active protein levels at the surface (predicting a higher clearance of rosuvastatin and thus lower plasma concentrations). This stresses the importance of considering individual differences in protein expression and alterations due to genetic variation for accurate modeling.

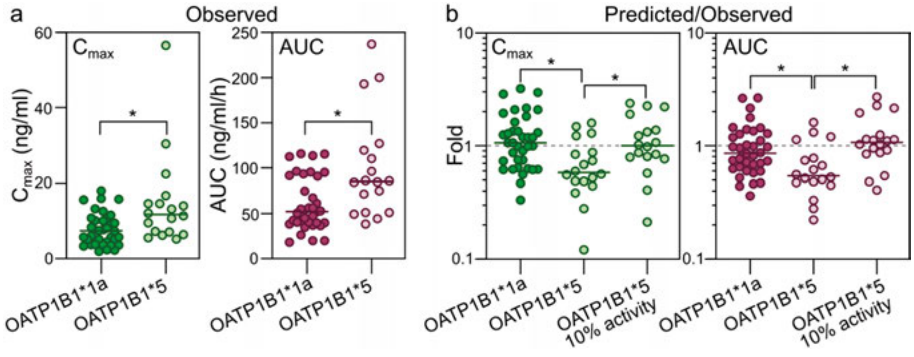

Figure 22. Pharmacokinetic parameters of rosuvastatin, separated by patients with SLCO1B1*1a and *5 variants. (**a**) Observed C_{max} and AUC-values from the time-dependent disposition of rosuvastatin in plasma. (**b**) Fold values from predicted and observed C_{max} and AUC-values from the model with optimized bile emptying rate, separated by patients with *1a and *5 variants before and after correcting for 10% activity of the *5 variant.[35]

Conclusions

This thesis provided improved understanding of variability in protein quantification as an effect of different mass spectrometry-based proteomics methodologies with different types of sample preparation procedures. Proteomics analyses offered explanations to differences in metabolic activity between two widely used *in vitro* systems: human liver microsomes and human hepatocytes. Further, this thesis contributed to an increased understanding of inter-individual differences in protein expression in human liver and jejunum. Proteomics analysis also provided increased knowledge in the effects of obesity, gastric bypass surgery, and weight loss on the liver and jejunal proteomes. Finally, proteomics scaling was used to create a mechanistic model from *in vitro* hepatic uptake kinetics, which successfully predicted the plasma distribution of rosuvastatin in 56 donors.

From the work presented herein, it can be concluded that:

- The largest variability in protein quantification could be ascribed to insufficient enrichment from subcellular fractionation processes, most likely due to the collection of the majority of proteins in the initial fraction of the differential centrifugation protocols.

- For compounds predominantly metabolized by a single enzyme, the faster microsomal metabolism could be explained by a higher available unbound drug concentration and CYP content in the microsomes as compared to in the hepatocytes.

- Proteins in human liver and jejunum covered a wide inter-individual variability spectrum, in which proteins with low expression variability were involved in essential cellular functions, while many proteins with high variability were related to disease.

- Patients with obesity that underwent gastric bypass surgery and subsequent weight loss showed lower levels of jejunal proteins involved in inflammatory response and drug metabolism.

- Parameters from *in vitro* kinetics and proteomics scaling could be used in prediction models of transporter-mediated clearance.

Future perspectives

In this thesis, mass spectrometry-based proteomics was used to increase the understanding of different *in vitro* systems and the effects of physiological and individual differences in tissue proteomics. The technique was also used to improve mechanistic models to predict drug disposition. In the proteomics approaches used in this thesis, proteins are measured directly from cell or tissue homogenates, without any purification steps to isolate specific types of proteins. This allows for the quantification of full proteomes. However, quantification of all proteins in the entire cell is not appropriate for all types of studies. For instance, in this thesis, the under-predictions observed for patients with the specific OATP1B1*5 variant were most likely caused by overestimation of the active transporter concentration (i.e., the transporter at the plasma membrane) in the livers. This overestimation could have been avoided if the proteomics method discriminated for proteins that were actually located on the plasma membrane, instead of also measuring the intra-cellular transporters. To study cell-surface proteins, subcellular fractionation is commonly used for isolating the plasma membrane proteins prior to analysis. However, this technique requires refinement for successful purification of plasma membrane fractions without substantial losses during the process. For this purpose, mass spectrometry-based proteomics could serve as a useful tool for evaluating the purity and the enrichment of proteins in the fractions. Other techniques used for quantifying surface proteins are based on chemical tagging (e.g., biotinylation) of proteins at the outer cell-surface, but are thus limited to cultured cells and not applicable to tissue samples. In an indirect approach, concentrations of surface proteins on the plasma membrane in tissues could be determined by scaling from cell assays. For this purpose, cell lines expressing transporters with a specific genetic variant could be used together with techniques that label the transporters at the surface or capture them with antibodies. By assuming that the stoichiometry of the transporter at the surface and inside the cell is the same in the cell lines as in the tissues, the ratio of surface and intracellular protein concentrations from the cell assay could potentially be used as a scaling factor for the surface protein concentration in the liver.

Variability in cell composition is common in biopsies from tissues comprising various cell types. For instance, since the jejunum is composed of several cell layers, it can be difficult to sample only the mucosa layer. Thus, jejunum biopsies can contain different proportions of epithelial cells and entero-

cytes that can confound the proteomics analysis by diluting the enterocyte proteins. To study the proteomes of only enterocytes, the cells could be isolated prior to the analysis. Another possibility is to use the obtained proteomics data and deconvolute the contribution of each different cell type. This could be made possible by establishing specific cell type protein markers that can be used to calculate the proportion of the respective cell type in the samples. With this information, protein concentrations could be adjusted based on the proportion of specific cells in the sample. Another approach would be to analyze single cells instead of the bulk of cells as is common today. This would also allow for studying inter-cell protein variance in cells obtained from different regions of the tissue. Currently, single-cell analysis is readily performed on the mRNA level, and with improved technology, single-cell proteomics is an emerging technique.

In this thesis, uptake kinetics parameters were scaled using proteomics data from different individuals to predict plasma drug distribution. However, other individual biological differences reflected in kidney and liver function, blood volume and pressure, and intestinal absorption can also influence the drug disposition. To assess the impact of such biological differences on drug efficacy and toxicity, physiologically-based pharmacokinetic (PBPK) models can be useful. This type of models include more physiological parameters and predict the drug disposition throughout the whole body, compared to the more simplistic model used in this thesis. More individualized predictions could be performed by incorporating information of clinical parameters and protein expression of different tissues from different patients. To further deepen the understanding of changes in biological processes and how they affect drug disposition, proteomics analysis could be combined with information from other 'omics' approaches. By combining the information from proteomics with genomics data, the effects of different genotypes could be investigated. Furthermore, metabolomics studies, in which metabolites created from different biological processes are analyzed, could provide improved understanding of the metabolic processes in patients. This integrated systems biology approach would enable a more holistic view of biological processes in the human body. By further incorporating drug pharmacokinetics and individual expression levels, quantitative systems pharmacology could contribute to improved drug development and personalized medicine.

Svensk populärvetenskaplig sammanfattning

De flesta läkemedel ges i form av tabletter via munnen. Läkemedlet färdas då genom mag-tarmkanalen för att kunna tas upp i blodet och vidare ut till det tilltänkta verkningsstället. Under denna färd finns det många steg som kan bestämma hur stor del av läkemedlet som når sitt mål. Läkemedlet måste först ta sig genom tunntarmens slemhinna för att kunna nå blodet. De flesta läkemedel avlägsnas sedan från kroppen genom omvandling (metabolism) i levern och utsöndring via galla eller urin, innan det hunnit komma till sitt mål. I kroppens celler finns molekyler som utför olika funktioner, så kallade proteiner. Olika typer av proteiner påverkar till stor del hur mycket av läkemedlet som tas upp i de olika organen (av transportörer) samt hur de omvandlas (av enzymer). Dessa proteiner finns i olika mängd i olika organ, och kan fungera olika effektivt för olika läkemedel. För att få en uppfattning av hur effektivt ett läkemedel är kan man i laboratoriemiljö bestämma nivåer och sammansättning av proteinerna i olika organ (med så kallad proteomik) och använda cellbaserade modeller (*in vitro*-system) som efterliknar de processer som sker i mänsklig tunntarm och lever för att undersöka hur effektivt läkemedlet förflyttas eller metaboliseras (genom aktivitetsstudier).

Målet med denna avhandling var att med hjälp av proteomik och aktivitetsstudier öka förståelsen av hur sådana *in vitro*-system fungerar och hur de kan användas för att förutspå hur ett läkemedel omsätts i kroppen. Vidare var målet att med hjälp av proteomik förstå hur läkemedelsomsättningen förändras på grund av kraftig övervikt och gastric bypass-operation, som används för att reducera födoupptaget hos överviktiga individer.

Arbetet började med att undersöka olika faktorer som kan påverka mätningen av mängden protein, genom att jämföra resultaten från olika proteomikmetoder. Den största variationen i proteinbestämningen erhölls från metoder som använde en metod för att öka koncentrationen av proteinerna för att underlätta analysen (så kallad subcellulär fraktionering). Dessa variationer uppstod troligtvis på grund av att stora mängder av proteinerna tappades under processen, vilket gav lägre nivåer av proteinerna än väntat i de prover som analyserades.

Sedan undersöktes skillnader mellan två *in vitro* system som är vanligt använda som modeller för att studera läkemedelsmetabolism i levern, så kallade mikrosomer och hepatocyter. Genom att använda proteomik och aktivitetsstudier, kunde skillnader i de två systemen förklaras av olika nivåer av enzymer och av hur mycket av läkemedlet som var tillgängligt i de respektive systemen.

Därefter studerades skillnader i proteinnivåer i mänsklig tunntarm och lever mellan olika individer. Denna studie visade att vissa proteiner hade jämförbara nivåer i de flesta individer medan andra skiljdes åt mycket. De proteiner som var jämförbara hade funktioner som var viktiga för cellers och därmed organs överlevnad, medan många av de som varierade mellan individer var relaterade till olika typer av sjukdomstillstånd.
Sedan undersöktes hur kraftig övervikt, gastric bypass-operation och viktnedgång påverkar proteinnivåerna i mänsklig tunntarm och lever. Efter gastric bypass-operation och medföljande viktnedgång hade patienterna lägre nivåer av proteiner som reglerar inflammation och läkemedelsmetabolism i tunntarmen.

Till sist användes proteomikdata från patienter, med eller utan kraftig övervikt, tillsammans med transportörsaktivitetsdata från *in vitro* för att skapa en modell som skulle förutspå läkemedelsfördelning och omsättning i kroppen. Resultatet visade att modellen kunde användas till att förutspå hur det kolesterolsänkande läkemedlet rosuvastatin fördelades i blodet hos patienterna.

Sammanfattningsvis bidrog denna avhandling med ökad förståelse för faktorer som påverkar bestämning av proteinkoncentrationer samt funktioner i *in vitro* system. Avhandlingen visar hur proteomik kan användas för att förstå skillnader som kan uppstå på grund av olika fysiologiska förändringar och på grund av olikheter i individer, samt hur proteomik kan användas i modeller för att förutspå läkemedelsomsättning i kroppen.

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References

1. Davis, S. S., Hardy, J. G. & Fara, J. W., *Transit of pharmaceutical dosage forms through the small intestine*. Gut, 1986. **27**(8): p. 886-92.
2. Teitelbaum, E. N., Vaziri, K., Zettervall, S., Amdur, R. L. & Orkin, B. A., *Intraoperative small bowel length measurements and analysis of demographic predictors of increased length*. Clinical Anatomy, 2013. **26**(7): p. 827-32.
3. Feher, J. in *Quantitative Human Physiology (Second Edition)* (ed Joseph Feher) 796-809 (Academic Press, 2017).
4. Turner, J. R. in *Epithelia and Gastrointestinal Function in Yamada' s Textbook of Gastroenterology* (ed Camilleri M. Podolsky D. K., Fitz J. G., Kalloo A. N., Shanahan F. and Wang T. C.) 317-29 (2015).
5. Montalban-Arques, A., Chaparro, M., Gisbert, J. P. & Bernardo, D., *The Innate Immune System in the Gastrointestinal Tract: Role of Intraepithelial Lymphocytes and Lamina Propria Innate Lymphoid Cells in Intestinal Inflammation*. Inflammatory Bowel Diseases, 2018. **24**(8): p. 1649-59.
6. Noah, T. K., Donahue, B. & Shroyer, N. F., *Intestinal development and differentiation*. Exp Cell Res, 2011. **317**(19): p. 2702-10.
7. Iqbal, J. & Hussain, M. M., *Intestinal lipid absorption*. Am J Physiol Endocrinol Metab, 2009. **296**(6): p. E1183-E94.
8. Lee, B., Moon, K. M. & Kim, C. Y., *Tight Junction in the Intestinal Epithelium: Its Association with Diseases and Regulation by Phytochemicals*. Journal of Immunology Research, 2018. **2018**: p. 11.
9. Molina, D. K. & DiMaio, V. J. M., *Normal Organ Weights in Men: Part II—The Brain, Lungs, Liver, Spleen, and Kidneys*. The American Journal of Forensic Medicine and Pathology, 2012. **33**(4): p. 368-72.
10. Grant, D. M., *Detoxification pathways in the liver*. Journal of Inherited Metabolic Disease, 1991. **14**(4): p. 421-30.
11. Abdel-Misih, S. R. Z. & Bloomston, M., *Liver anatomy*. Surg Clin North Am, 2010. **90**(4): p. 643-53.
12. Roberts, M. S., Magnusson, B. M., Burczynski, F. J. & Weiss, M., *Enterohepatic Circulation*. Clinical Pharmacokinetics, 2002. **41**(10): p. 751-90.
13. Trefts, E., Gannon, M. & Wasserman, D. H., *The liver*. Current Biology, 2017. **27**(21): p. R1147-R51.
14. Stanger, B. Z., *Cellular homeostasis and repair in the mammalian liver*. Annual review of physiology, 2015. **77**: p. 179-200.

15. Cox, J. & Mann, M., *Quantitative, High-Resolution Proteomics for Data-Driven Systems Biology*. Annual Review of Biochemistry, 2011. **80**(1): p. 273-99.
16. Altelaar, A. F. M., Munoz, J. & Heck, A. J. R., *Next-generation proteomics: towards an integrative view of proteome dynamics*. Nature Reviews Genetics, 2012. **14**: p. 35-48.
17. Manzoni, C. *et al.*, *Genome, transcriptome and proteome: the rise of omics data and their integration in biomedical sciences*. Briefings in Bioinformatics, 2016. **19**(2): p. 286-302.
18. Towbin, H., Staehelin, T. & Gordon, J., *Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications*. Proc Natl Acad Sci U S A, 1979. **76**(9): p. 4350-54.
19. Mahmood, T. & Yang, P.-C., *Western blot: technique, theory, and trouble shooting*. N Am J Med Sci, 2012. **4**(9): p. 429-34.
20. Michel, M. C., Wieland, T. & Tsujimoto, G., *How reliable are G-protein-coupled receptor antibodies?* Naunyn-Schmiedeberg's Archives of Pharmacology, 2009. **379**(4): p. 385-88.
21. Bass, J. J. *et al.*, *An overview of technical considerations for Western blotting applications to physiological research*. Scand J Med Sci Sports, 2017. **27**(1): p. 4-25.
22. Lequin, R. M., *Enzyme Immunoassay (EIA)/Enzyme-Linked Immunosorbent Assay (ELISA)*. Clinical Chemistry, 2005. **51**(12): p. 2415.
23. Kaur, H., Bhagwat, S. R., Sharma, T. K. & Kumar, A., *Analytical techniques for characterization of biological molecules – proteins and aptamers/oligonucleotides*. Bioanalysis, 2018. **11**(2): p. 103-17.
24. Aebersold, R. & Mann, M., *Mass-spectrometric exploration of proteome structure and function*. Nature, 2016. **537**: p. 347.
25. Gillet, L. C., Leitner, A. & Aebersold, R., *Mass Spectrometry Applied to Bottom-Up Proteomics: Entering the High-Throughput Era for Hypothesis Testing*. Annual Review of Analytical Chemistry, 2016. **9**(1): p. 449-72.
26. Lodish, H. *et al.* in *Molecular Cell Biology. 4th edition* (WH Freeman, 2000).
27. Harwood, M. D., Russell, M. R., Neuhoff, S., Warhurst, G. & Rostami-Hodjegan, A., *Lost in Centrifugation: Accounting for Transporter Protein Losses in Quantitative Targeted Absolute Proteomics*. Drug Metabolism and Disposition, 2014. **42**(10): p. 1766.
28. Cox, B. & Emili, A., *Tissue subcellular fractionation and protein extraction for use in mass-spectrometry-based proteomics*. Nature Protocols, 2006. **1**(4): p. 1872-78.
29. Weekes, M. P. *et al.*, *Comparative analysis of techniques to purify plasma membrane proteins*. J Biomol Tech, 2010. **21**(3): p. 108-15.

30. Mei, H. *et al.*, *Investigation of matrix effects in bioanalytical high-performance liquid chromatography/tandem mass spectrometric assays: application to drug discovery*. Rapid Communications in Mass Spectrometry, 2003. **17**(1): p. 97-103.
31. Rogers, J. C. & Bomgarden, R. D. in *Modern Proteomics – Sample Preparation, Analysis and Practical Applications* (eds Hamid Mirzaei & Martin Carrasco) 43-62 (Springer International Publishing, 2016).
32. Annesley, T. M., *Ion Suppression in Mass Spectrometry*. Clinical Chemistry, 2003. **49**(7): p. 1041-44.
33. Wiśniewski, J. R., Zougman, A., Nagaraj, N. & Mann, M., *Universal sample preparation method for proteome analysis*. Nature Methods, 2009. **6**: p. 359.
34. Hughes, C. S. *et al.*, *Ultrasensitive proteome analysis using paramagnetic bead technology*. Molecular systems biology, 2014. **10**(10): p. 757-57.
35. Ho, R. H. *et al.*, *Drug and bile acid transporters in rosuvastatin hepatic uptake: function, expression, and pharmacogenetics*. Gastroenterology, 2006. **130**(6): p. 1793-806.
36. Olsen, J. V., Ong, S.-E. & Mann, M., *Trypsin Cleaves Exclusively C-terminal to Arginine and Lysine Residues*. Molecular & Cellular Proteomics, 2004. **3**(6): p. 608.
37. Glatter, T. *et al.*, *Large-Scale Quantitative Assessment of Different In-Solution Protein Digestion Protocols Reveals Superior Cleavage Efficiency of Tandem Lys-C/Trypsin Proteolysis over Trypsin Digestion*. J Proteome Res, 2012. **11**(11): p. 5145-56.
38. Wiśniewski, J. R. & Rakus, D., *Multi-enzyme digestion FASP and the 'Total Protein Approach'-based absolute quantification of the Escherichia coli proteome*. Journal of Proteomics, 2014. **109**: p. 322-31.
39. Yang, F., Shen, Y., Camp, D. G., 2nd & Smith, R. D., *High-pH reversed-phase chromatography with fraction concatenation for 2D proteomic analysis*. Expert Rev Proteomics, 2012. **9**(2): p. 129-34.
40. Branca, R. M. M. *et al.*, *HiRIEF LC-MS enables deep proteome coverage and unbiased proteogenomics*. Nature Methods, 2013. **11**: p. 59.
41. Weiß, F. *et al.*, *Indirect protein quantification of drug-transforming enzymes using peptide group-specific immunoaffinity enrichment and mass spectrometry*. Scientific Reports, 2015. **5**: p. 8759.
42. Wilkins, M. R. *et al.*, *From proteins to proteomes: large scale protein identification by two-dimensional electrophoresis and arnino acid analysis*. Bio/technology, 1996. **14**(1): p. 61.
43. Vidova, V. & Spacil, Z., *A review on mass spectrometry-based quantitative proteomics: Targeted and data independent acquisition*. Analytica Chimica Acta, 2017. **964**: p. 7-23.

44. Prasad, B. *et al.*, *Towards a Consensus on Applying Quantitative LC-MS/MS Proteomics in Translational Pharmacology Research: A White Paper*. Clinical Pharmacology & Therapeutics, 2019. **0**(ja): p.
45. Brownridge, P. J., Harman, V. M., Simpson, D. M. & Beynon, R. J. in *Quantitative Methods in Proteomics* (ed Katrin Marcus) 267-93 (Humana Press, 2012).
46. Rowland, M., Peck, C. & Tucker, G., *Physiologically-Based Pharmacokinetics in Drug Development and Regulatory Science*. Annual Review of Pharmacology and Toxicology, 2011. **51**(1): p. 45-73.
47. Zhuang, X. & Lu, C., *PBPK modeling and simulation in drug research and development*. Acta Pharm Sin B, 2016. **6**(5): p. 430-40.
48. Bosgra, S. *et al.*, *Predicting carrier-mediated hepatic disposition of rosuvastatin in man by scaling from individual transfected cell-lines in vitro using absolute transporter protein quantification and PBPK modeling*. European Journal of Pharmaceutical Sciences, 2014. **65**: p. 156-66.
49. Kunze, A., Huwyler, J., Camenisch, G. & Poller, B., *Prediction of Organic Anion-Transporting Polypeptide 1B1- and 1B3-Mediated Hepatic Uptake of Statins Based on Transporter Protein Expression and Activity Data*. Drug Metabolism and Disposition, 2014. **42**(9): p. 1514.
50. Tyanova, S., Temu, T. & Cox, J., *The MaxQuant computational platform for mass spectrometry-based shotgun proteomics*. Nature Protocols, 2016. **11**: p. 2301.
51. Koopmans, F., Ho, J. T. C., Smit, A. B. & Li, K. W., *Comparative Analyses of Data Independent Acquisition Mass Spectrometric Approaches: DIA, WiSIM-DIA, and Untargeted DIA*. Proteomics, 2018. **18**(1): p. 1700304.
52. Rauniyar, N. & Yates, J. R., 3rd. *Isobaric labeling-based relative quantification in shotgun proteomics*. J Proteome Res, 2014. **13**(12): p. 5293-309.
53. Schwanhäusser, B. *et al.*, *Global quantification of mammalian gene expression control*. Nature, 2011. **473**: p. 337.
54. Vildhede, A. *et al.*, *Comparison of proteomic quantification approaches for hepatic drug transporters: multiplexed global quantitation correlates with targeted proteomic quantitation*. Drug Metabolism and Disposition, 2018. **46**(5): p. 692-96.
55. Vildhede, A., Wiśniewski, J. R., Norén, A., Karlgren, M. & Artursson, P., *Comparative Proteomic Analysis of Human Liver Tissue and Isolated Hepatocytes with a Focus on Proteins Determining Drug Exposure*. J Proteome Res, 2015. **14**(8): p. 3305-14.
56. Ölander, M. *Proteomic and Functional Analysis of In Vitro Systems for Studies of Drug Disposition in the Human Small Intestine and Liver*, Acta Universitatis Upsaliensis, (2019).

57. Ölander, M. *et al.*, *A simple approach for restoration of differentiation and function in cryopreserved human hepatocytes*. Archives of Toxicology, 2019. **93**(3): p. 819-29.
58. Wang, D. *et al.*, *A deep proteome and transcriptome abundance atlas of 29 healthy human tissues*. Molecular systems biology, 2019. **15**(2): p. e8503.
59. Wewer Albrechtsen, N. J. *et al.*, *Plasma Proteome Profiling Reveals Dynamics of Inflammatory and Lipid Homeostasis Markers after Roux-En-Y Gastric Bypass Surgery*. Cell Systems, 2018. **7**(6): p. 601-12.e3.
60. Vildhede, A. *et al.*, *Hepatic Uptake of Atorvastatin: Influence of Variability in Transporter Expression on Uptake Clearance and Drug-Drug Interactions*. Drug Metabolism and Disposition, 2014. **42**(7): p. 1210.
61. Vildhede, A. *et al.*, *Mechanistic Modeling of Pitavastatin Disposition in Sandwich-Cultured Human Hepatocytes: A Proteomics-Informed Bottom-Up Approach*. Drug Metabolism and Disposition, 2016. **44**(4): p. 505.
62. Brodin, B., Steffansen, B. & Nielsen, C. U., *Passive diffusion of drug substances: the concepts of flux and permeability*. Molecular Biopharmaceutics, 2010. p. 135-52.
63. Liu, X., Testa, B. & Fahr, A., *Lipophilicity and Its Relationship with Passive Drug Permeation*. Pharmaceutical Research, 2011. **28**(5): p. 962-77.
64. Xiang, T. X. & Anderson, B. D., *The relationship between permeant size and permeability in lipid bilayer membranes*. The Journal of Membrane Biology, 1994. **140**(2): p. 111-22.
65. Tamura, A. & Tsukita, S., *Paracellular barrier and channel functions of TJ claudins in organizing biological systems: Advances in the field of barriology revealed in knockout mice*. Seminars in Cell & Developmental Biology, 2014. **36**: p. 177-85.
66. Adson, A. *et al.*, *Passive Diffusion of Weak Organic Electrolytes across Caco□2 Cell Monolayers: Uncoupling the Contributions of Hydrodynamic, Transcellular, and Paracellular Barriers*. Journal of Pharmaceutical Sciences, 1995. **84**(10): p. 1197-204.
67. Sugano, K. *et al.*, *Coexistence of passive and carrier-mediated processes in drug transport*. Nature Reviews Drug Discovery, 2010. **9**: p. 597.
68. Smith, D. *et al.*, *Passive Lipoidal Diffusion and Carrier-Mediated Cell Uptake Are Both Important Mechanisms of Membrane Permeation in Drug Disposition*. Molecular Pharmaceutics, 2014. **11**(6): p. 1727-38.
69. You, G. a. M., M.E. in *Overview of Drug Transporter Families in Drug Transporters* 1-6 (2014).

70. Banerjee, A., Johnston, J. S. & Swaan, P. W. in *Cellular Drug Delivery: Principles and Practice* (eds D. Robert Lu & Svein Øie) 107-28 (Humana Press, 2004).
71. The International Transporter, C. *et al.*, *Membrane transporters in drug development*. Nature Reviews Drug Discovery, 2010. **9**: p. 215.
72. O'Hagan, S., Muelas, M. W., Day, P. J., Lundberg, E. & Kell, D. B., *GeneGini: Assessment via the Gini Coefficient of Reference "Housekeeping" Genes and Diverse Human Transporter Expression Profiles*. Cell Systems, 2018. **6**(2): p. 230-44. e1.
73. Nigam, S. K., *What do drug transporters really do?* Nature Reviews Drug Discovery, 2014. **14**: p. 29.
74. Zamek□Gliszczynski, M. J. *et al.*, *Transporters in drug development: 2018 ITC recommendations for transporters of emerging clinical importance*. Clinical Pharmacology & Therapeutics, 2018. **104**(5): p. 890-99.
75. Zhang, Z. & Tang, W., *Drug metabolism in drug discovery and development*. Acta Pharm Sin B, 2018. **8**(5): p. 721-32.
76. Iyanagi, T. in *International Review of Cytology* Vol. 260 35-112 (Academic Press, 2007).
77. Williams, J. A. *et al.*, *Drug-drug interactions for UDP-glucuronosyltransferase substrates: a pharmacokinetic explanation for typically observed low exposure (AUCi/AUC) ratios*. Drug Metabolism and Disposition, 2004. **32**(11): p. 1201-08.
78. Zanger, U. M. & Schwab, M., *Cytochrome P450 enzymes in drug metabolism: Regulation of gene expression, enzyme activities, and impact of genetic variation*. Pharmacology & Therapeutics, 2013. **138**(1): p. 103-41.
79. Drozdzik, M. *et al.*, *Protein Abundance of Clinically Relevant Drug□Metabolizing Enzymes in the Human Liver and Intestine: A Comparative Analysis in Paired Tissue Specimens*. Clinical Pharmacology & Therapeutics, 2018. **104**(3): p. 515-24.
80. Michael, B. F. & Gabriel, L., *The Role of the Intestine in Drug Metabolism and Pharmacokinetics: An Industry Perspective*. Current Drug Metabolism, 2007. **8**(7): p. 694-99.
81. Blüher, M., *Obesity: global epidemiology and pathogenesis*. Nature Reviews Endocrinology, 2019. **15**(5): p. 288-98.
82. Knibbe, C. A. J. *et al.*, *Drug disposition in obesity: toward evidence-based dosing*. Annual review of pharmacology and toxicology, 2015. **55**: p. 149-67.
83. Hanley, M. J., Abernethy, D. R. & Greenblatt, D. J., *Effect of Obesity on the Pharmacokinetics of Drugs in Humans*. Clinical Pharmacokinetics, 2010. **49**(2): p. 71-87.
84. Brill, M. J. E. *et al.*, *Impact of Obesity on Drug Metabolism and Elimination in Adults and Children*. Clinical Pharmacokinetics, 2012. **51**(5): p. 277-304.

85. Ulvestad, M. *et al.*, *Impact of OATP1B1, MDR1, and CYP3A4 Expression in Liver and Intestine on Interpatient Pharmacokinetic Variability of Atorvastatin in Obese Subjects*. Clinical Pharmacology & Therapeutics, 2013. **93**(3): p. 275-82.
86. Arterburn, D. E. & Courcoulas, A. P., *Bariatric surgery for obesity and metabolic conditions in adults*. bmj, 2014. **349**: p. g3961.
87. Kang, J. H. & Le, Q. A., *Effectiveness of bariatric surgical procedures: A systematic review and network meta-analysis of randomized controlled trials*. Medicine, 2017. **96**(46): p.
88. Cavin, J.-B. *et al.*, *Differences in Alimentary Glucose Absorption and Intestinal Disposal of Blood Glucose After Roux-en-Y Gastric Bypass vs Sleeve Gastrectomy*. Gastroenterology, 2016. **150**(2): p. 454-64.e9.
89. Spak, E. *et al.*, *Changes in the mucosa of the Roux-limb after gastric bypass surgery*. Histopathology, 2010. **57**(5): p. 680-88.
90. Volpe, D. A. & Balimane, P. V., *Application of in vitro CYP and transporter assays to predict clinical drug–drug interactions*. Bioanalysis, 2018. **10**(9): p. 619-23.
91. Andrade, E. L. *et al.*, *Non-clinical studies required for new drug development - Part I: early in silico and in vitro studies, new target discovery and validation, proof of principles and robustness of animal studies*. Braz J Med Biol Res, 2016. **49**(11): p. e5644-e44.
92. Johnson, K. A. & Goody, R. S., *The Original Michaelis Constant: Translation of the 1913 Michaelis–Menten Paper*. Biochemistry, 2011. **50**(39): p. 8264-69.
93. Michaelis, L. & Menten, M. L., *Die kinetik der invertinwirkung*. Biochemische Zeitschrift, 1913.(49): p. 333–69.
94. Houston, J. B. & Kenworthy, K. E., *In vitro-in vivo scaling of CYP kinetic data not consistent with the classical Michaelis-Menten model*. Drug Metabolism and Disposition, 2000. **28**(3): p. 246-54.
95. Korprasertthaworn, P. *et al.*, *In vitro characterization of the human liver microsomal kinetics and reaction phenotyping of olanzapine metabolism*. Drug Metabolism and Disposition, 2015. **43**(11): p. 1806-14.
96. Brown, H. S., Wilby, A. J., Alder, J. & Houston, J. B., *Comparative Use of Isolated Hepatocytes and Hepatic Microsomes for Cytochrome P450 Inhibition Studies: Transporter-Enzyme Interplay*. Drug Metabolism and Disposition, 2010. **38**(12): p. 2139-46.
97. Obach, R. S., *Prediction of Human Clearance of Twenty-Nine Drugs from Hepatic Microsomal Intrinsic Clearance Data: An Examination of In Vitro Half-Life Approach and Nonspecific Binding to Microsomes*. Drug Metabolism and Disposition, 1999. **27**(11): p. 1350-59.
98. Di, L. & Obach, R. S., *Addressing the challenges of low clearance in drug research*. AAPS J, 2015. **17**(2): p. 352-57.

99. Artursson, P., Palm, K. & Luthman, K., *Caco-2 monolayers in experimental and theoretical predictions of drug transport* Advanced Drug Delivery Reviews, 2001. **46**(1): p. 27-43.
100. Chen, E. C. *et al.*, *Evaluating the Utility of Canine Mdr1 Knockout Madin-Darby Canine Kidney I Cells in Permeability Screening and Efflux Substrate Determination*. Molecular Pharmaceutics, 2018. **15**(11): p. 5103-13.
101. Wu, C.-P., Calcagno, A. M. & Ambudkar, S. V., *Reversal of ABC drug transporter-mediated multidrug resistance in cancer cells: evaluation of current strategies*. Current molecular pharmacology, 2008. **1**(2): p. 93-105.
102. Kimoto, E. *et al.*, *Organic Anion Transporter 2–Mediated Hepatic Uptake Contributes to the Clearance of High-Permeability–Low-Molecular-Weight Acid and Zwitterion Drugs: Evaluation Using 25 Drugs*. Journal of Pharmacology and Experimental Therapeutics, 2018. **367**(2): p. 322.
103. Glavinas, H. *et al.*, *Utilization of membrane vesicle preparations to study drug–ABC transporter interactions*. Expert opinion on drug metabolism & toxicology, 2008. **4**(6): p. 721-32.
104. Li, A. P., *Evaluation of Adverse Drug Properties with Cryopreserved Human Hepatocytes and the Integrated Discrete Multiple Organ Co-culture (IdMOC(TM)) System*. Toxicol Res, 2015. **31**(2): p. 137-49.
105. LeCluyse, E. L. & Alexandre, E. in *Hepatocytes: Methods and Protocols* (ed Patrick Maurel) 57-82 (Humana Press, 2010).
106. Griffin, S. J. & Houston, J. B., *Prediction of in vitro intrinsic clearance from hepatocytes: comparison of suspensions and monolayer cultures*. Drug Metabolism and Disposition, 2005. **33**(1): p. 115-20.
107. Elaut, G. *et al.*, *Molecular mechanisms underlying the dedifferentiation process of isolated hepatocytes and their cultures*. Current drug metabolism, 2006. **7**(6): p. 629-60.
108. Deferm, N. *et al.* in *Experimental Cholestasis Research* (ed Mathieu Vinken) 335-50 (Springer New York, 2019).
109. Deharde, D. *et al.*, *Bile canaliculi formation and biliary transport in 3D sandwich-cultured hepatocytes in dependence of the extracellular matrix composition*. Archives of Toxicology, 2016. **90**(10): p. 2497-511.
110. Vorrink, S. U., Zhou, Y., Ingelman-Sundberg, M. & Lauschke, V. M., *Prediction of Drug-Induced Hepatotoxicity Using Long-Term Stable Primary Hepatic 3D Spheroid Cultures in Chemically Defined Conditions*. Toxicological Sciences, 2018. **163**(2): p. 655-65.
111. Bell, C. C. *et al.*, *Characterization of primary human hepatocyte spheroids as a model system for drug-induced liver injury, liver function and disease*. Scientific Reports, 2016. **6**: p. 25187.

112. Jia, L. & Liu, X., *The conduct of drug metabolism studies considered good practice (II): in vitro experiments*. Current drug metabolism, 2007. **8**(8): p. 822-29.
113. Hausjell, J., Halbwirth, H. & Spadiut, O., *Recombinant production of eukaryotic cytochrome P450s in microbial cell factories*. Biosci Rep, 2018. **38**(2): p. BSR20171290.
114. Alberts, B. *et al.* in *Molecular Biology of the Cell. 4th edition* (Garland Science, 2002).
115. Andringa, K. K. & Bailey, S. M. in *Methods in Enzymology* Vol. 474 (eds Enrique Cadenas & Lester Packer) 83-108 (Academic Press, 2010).
116. Boobis, A. R. *et al.*, *Monooxygenase activity of human liver in microsomal fractions of needle biopsy specimens*. British Journal of Clinical Pharmacology, 1980. **9**(1): p. 11-19.
117. Graham, J., *Preparation of Crude Subcellular Fractions by Differential Centrifugation* TheScientificWorldJournal, 2002. **2**: p.
118. Hayes, K. A., Brennan, B., Chenery, R. & Houston, J. B., *In vivo disposition of caffeine predicted from hepatic microsomal and hepatocyte data*. Drug Metabolism and Disposition, 1995. **23**(3): p. 349-53.
119. Hermann, M., Kase, E. T., Molden, E. & Christensen, H., *Evaluation of microsomal incubation conditions on CYP3A4-mediated metabolism of cyclosporine A by a statistical experimental design*. Current drug metabolism, 2006. **7**(3): p. 265-71.
120. Palade, G. E. & Siekevitz, P., *Liver microsomes*. The Journal of Biophysical and Biochemical Cytology, 1956. **2**(2): p. 171.
121. Zhang, H. *et al.*, *Content and activity of human liver microsomal protein and prediction of individual hepatic clearance in vivo*. Scientific Reports, 2015. **5**: p. 17671.
122. Fisher, M. B., Campanale, K., Ackermann, B. L., VandenBranden, M. & Wrighton, S. A., *In vitro glucuronidation using human liver microsomes and the pore-forming peptide alamethicin*. Drug metabolism and disposition, 2000. **28**(5): p. 560-66.
123. Cederbaum, A. I., *Molecular mechanisms of the microsomal mixed function oxidases and biological and pathological implications*. Redox Biol, 2015. **4**: p. 60-73.
124. Obach, R. S., Walsky, R. L. & Venkatakrishnan, K., *Mechanism-Based Inactivation of Human Cytochrome P450 Enzymes and the Prediction of Drug-Drug Interactions*. Drug Metabolism and Disposition, 2007. **35**(2): p. 246-55.
125. Hakooz, N. *et al.*, *Determination of a Human Hepatic Microsomal Scaling Factor for Predicting in Vivo Drug Clearance*. Pharmaceutical Research, 2006. **23**(3): p. 533-39.
126. Shah, P. *et al.*, *An Automated High-Throughput Metabolic Stability Assay Using an Integrated High-Resolution Accurate Mass Method*

and Automated Data Analysis Software. Drug Metab Dispos, 2016. **44**(10): p. 1653-61.
127. Smith, C. M. *et al.*, *A comprehensive evaluation of metabolic activity and intrinsic clearance in suspensions and monolayer cultures of cryopreserved primary human hepatocytes*. Journal of Pharmaceutical Sciences, 2012. **101**(10): p. 3989-4002.
128. Sohlenius-Sternbeck, A. K. *et al.*, *Evaluation of the human prediction of clearance from hepatocyte and microsome intrinsic clearance for 52 drug compounds*. Xenobiotica, 2010. **40**(9): p. 637-49.
129. Di, L. *et al.*, *Mechanistic insights from comparing intrinsic clearance values between human liver microsomes and hepatocytes to guide drug design*. European Journal of Medicinal Chemistry, 2012. **57**: p. 441-48.
130. Treyer, A. *et al.*, *Intracellular Drug Bioavailability: Effect of Neutral Lipids and Phospholipids*. Molecular Pharmaceutics, 2018. **15**(6): p. 2224-33.
131. Yoshikado, T. *et al.*, *Comparison of Methods for Estimating Unbound Intracellular-to-Medium Concentration Ratios in Rat and Human Hepatocytes Using Statins*. Drug Metabolism and Disposition, 2017. **45**(7): p. 779.
132. Mateus, A., Matsson, P. & Artursson, P., *Rapid Measurement of Intracellular Unbound Drug Concentrations*. Molecular Pharmaceutics, 2013. **10**(6): p. 2467-78.
133. Filppula, A. M., Parvizi, R., Mateus, A., Baranczewski, P. & Artursson, P., *Improved predictions of time-dependent drug-drug interactions by determination of cytosolic drug concentrations*. Scientific Reports, 2019. **9**(1): p. 5850.
134. Mateus, A. *et al.*, *Prediction of intracellular exposure bridges the gap between target- and cell-based drug discovery*. Proceedings of the National Academy of Sciences, 2017. **114**(30): p. E6231.
135. Mateus, A. *et al.*, *Intracellular drug bioavailability: a new predictor of system dependent drug disposition*. Scientific Reports, 2017. **7**: p. 43047.
136. Treyer, A. *et al.*, *Impact of Intracellular Concentrations on Metabolic Drug-Drug Interaction Studies*. AAPS J, 2019. **21**(5): p. 77.
137. Aarons, L., *Physiologically based pharmacokinetic modelling: a sound mechanistic basis is needed*. British journal of clinical pharmacology, 2005. **60**(6): p. 581-83.
138. Tsamandouras, N., Rostami-Hodjegan, A. & Aarons, L., *Combining the 'bottom up' and 'top down' approaches in pharmacokinetic modelling: fitting PBPK models to observed clinical data*. British journal of clinical pharmacology, 2015. **79**(1): p. 48-55.
139. Brown, H. S., Griffin, M. & Houston, J. B., *Evaluation of Cryopreserved Human Hepatocytes as an Alternative in Vitro System*

to Microsomes for the Prediction of Metabolic Clearance. Drug Metabolism and Disposition, 2007. **35**(2): p. 293-301.
140. Riley, R. J., McGinnity, D. F. & Austin, R. P., *A unified model for predicting human hepatic, metabolic clearance from in vitro clearance data in hepatocytes and microsomes*. Drug Metabolism and Disposition, 2005. **33**(9): p. 1304-11.
141. Li, N., Bi, Y.-A., Duignan, D. B. & Lai, Y., *Quantitative Expression Profile of Hepatobiliary Transporters in Sandwich Cultured Rat and Human Hepatocytes*. Molecular Pharmaceutics, 2009. **6**(4): p. 1180-89.
142. Barter, Z. E. *et al.*, *Scaling factors for the extrapolation of in vivo metabolic drug clearance from in vitro data: reaching a consensus on values of human micro-somal protein and hepatocellularity per gram of liver*. Current drug metabolism, 2007. **8**(1): p. 33-45.
143. Houston, B. J., *Utility of in vitro drug metabolism data in predicting in vivo metabolic clearance*. Biochemical Pharmacology, 1994. **47**(9): p. 1469-79.
144. Lipscomb, J. C., Teuschler, L. K., Swartout, J. C., Striley, C. A. F. & Snawder, J. E., *Variance of Microsomal Protein and Cytochrome P450 2E1 and 3A Forms in Adult Human Liver*. Toxicology Mechanisms and Methods, 2003. **13**(1): p. 45-51.
145. Wilson, Z. E. *et al.*, *Inter-individual variability in levels of human microsomal protein and hepatocellularity per gram of liver*. British journal of clinical pharmacology, 2003. **56**(4): p. 433-40.
146. Carlile, D. J., Zomorodi, K. & Houston, J. B., *Scaling Factors to Relate Drug Metabolic Clearance in Hepatic Microsomes, Isolated Hepatocytes, and the Intact Liver*. Drug Metabolism and Disposition, 1997. **25**(8): p. 903-11.
147. Li, N., Singh, P., Mandrell, K. M. & Lai, Y., *Improved Extrapolation of Hepatobiliary Clearance from in Vitro Sandwich Cultured Rat Hepatocytes through Absolute Quantification of Hepatobiliary Transporters*. Molecular Pharmaceutics, 2010. **7**(3): p. 630-41.
148. Akazawa, T., Uchida, Y., Tachikawa, M., Ohtsuki, S. & Terasaki, T., *Quantitative Targeted Absolute Proteomics of Transporters and Pharmacoproteomics-Based Reconstruction of P-Glycoprotein Function in Mouse Small Intestine*. Molecular Pharmaceutics, 2016. **13**(7): p. 2443-56.
149. Rose, R. H. *et al.*, *Application of a Physiologically Based Pharmacokinetic Model to Predict OATP1B1-Related Variability in Pharmacodynamics of Rosuvastatin*. CPT: Pharmacometrics & Systems Pharmacology, 2014. **3**(7): p. 124.
150. Hirano, M., Maeda, K., Shitara, Y. & Sugiyama, Y., *Contribution of OATP2 (OATP1B1) and OATP8 (OATP1B3) to the Hepatic Uptake of Pitavastatin in Humans*. Journal of Pharmacology and Experimental Therapeutics, 2004. **311**(1): p. 139.

151. Wiśniewski, J. R. & Mann, M., *Consecutive Proteolytic Digestion in an Enzyme Reactor Increases Depth of Proteomic and Phosphoproteomic Analysis*. Analytical Chemistry, 2012. **84**(6): p. 2631-37.
152. Wiśniewski, J. R. & Gaugaz, F. Z., *Fast and Sensitive Total Protein and Peptide Assays for Proteomic Analysis*. Analytical Chemistry, 2015. **87**(8): p. 4110-16.
153. Gröer, C. *et al.*, *LC–MS/MS-based quantification of clinically relevant intestinal uptake and efflux transporter proteins*. Journal of Pharmaceutical and Biomedical Analysis, 2013. **85**: p. 253-61.
154. Tyanova, S. *et al.*, *The Perseus computational platform for comprehensive analysis of (prote)omics data*. Nature Methods, 2016. **13**: p. 731.
155. Ritchie, M. E. *et al.*, *limma powers differential expression analyses for RNA-sequencing and microarray studies*. Nucleic acids research, 2015. **43**(7): p. e47-e47.
156. Huang, D. W., Sherman, B. T. & Lempicki, R. A., *Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources*. Nature protocols, 2009. **4**(1): p. 44.
157. Thul, P. J. *et al.*, *A subcellular map of the human proteome*. Science, 2017. **356**(6340): p. eaal3321.
158. Jadot, M. *et al.*, *Accounting for Protein Subcellular Localization: A Compartmental Map of the Rat Liver Proteome*. Molecular & cellular proteomics, 2017. **16**(2): p. 194-212.
159. Gillen, C. M. & Forbush Iii, B., *Functional interaction of the K-Cl cotransporter (KCC1) with the Na-K-Cl cotransporter in HEK-293 cells*. American Journal of Physiology-Cell Physiology, 1999. **276**(2): p. C328-C36.
160. Karlgren, M. *et al.*, *Classification of Inhibitors of Hepatic Organic Anion Transporting Polypeptides (OATPs): Influence of Protein Expression on Drug–Drug Interactions*. Journal of Medicinal Chemistry, 2012. **55**(10): p. 4740-63.
161. Ishida, K., Ullah, M., Tóth, B., Juhasz, V. & Unadkat, J. D., *Successful Prediction of In Vivo Hepatobiliary Clearances and Hepatic Concentrations of Rosuvastatin Using Sandwich-Cultured Rat Hepatocytes, Transporter-Expressing Cell Lines, and Quantitative Proteomics*. Drug Metabolism and Disposition, 2018. **46**(1): p. 66.
162. Martin, P. D., Warwick, M. J., Dane, A. L., Brindley, C. & Short, T., *Absolute oral bioavailability of rosuvastatin in healthy white adult male volunteers*. Clinical Therapeutics, 2003. **25**(10): p. 2553-63.
163. Jamei, M. *et al.*, *A mechanistic framework for in vitro–in vivo extrapolation of liver membrane transporters: prediction of drug–drug interaction between rosuvastatin and cyclosporine*. Clinical pharmacokinetics, 2014. **53**(1): p. 73-87.

164. Jones, H. M. & Rowland-Yeo, K., *Basic Concepts in Physiologically Based Pharmacokinetic Modeling in Drug Discovery and Development*. CPT: Pharmacometrics & Systems Pharmacology, 2013. **2**(8): p. 63.
165. Rodgers, T. & Rowland, M., *Physiologically based pharmacokinetic modelling 2: Predicting the tissue distribution of acids, very weak bases, neutrals and zwitterions*. Journal of Pharmaceutical Sciences, 2006. **95**(6): p. 1238-57.
166. Rodgers, T. & Rowland, M., *Mechanistic Approaches to Volume of Distribution Predictions: Understanding the Processes*. Pharmaceutical Research, 2007. **24**(5): p. 918-33.
167. Vezina, W. C. *et al.*, *Increased volume and decreased emptying of the gallbladder in large (Morbidly obese, tall normal, and muscular normal) people*. Gastroenterology, 1990. **98**(4): p. 1000-07.
168. Abe, K., Bridges, A. S. & Brouwer, K. L. R., *Use of sandwich-cultured human hepatocytes to predict biliary clearance of angiotensin II receptor blockers and HMG-CoA reductase inhibitors*. Drug Metab Dispos, 2009. **37**(3): p. 447-52.
169. Dobson, L., Reményi, I. & Tusnády, G. E., *The human transmembrane proteome*. Biol Direct, 2015. **10**: p. 31-31.
170. Fagerberg, L., Jonasson, K., von Heijne, G., Uhlén, M. & Berglund, L., *Prediction of the human membrane proteome*. PROTEOMICS, 2010. **10**(6): p. 1141-49.
171. Itzhak, D. N., Tyanova, S., Cox, J. & Borner, G. H. H., *Global, quantitative and dynamic mapping of protein subcellular localization*. eLife, 2016. **5**: p. e16950.
172. Wiśniewski, J. R., Duś, K. & Mann, M., *Proteomic workflow for analysis of archival formalin-fixed and paraffin-embedded clinical samples to a depth of 10 000 proteins*. PROTEOMICS – Clinical Applications, 2013. **7**(3-4): p. 225-33.
173. Ohtsuki, S. *et al.*, *Quantitative Targeted Absolute Proteomic Analysis of Transporters, Receptors and Junction Proteins for Validation of Human Cerebral Microvascular Endothelial Cell Line hCMEC/D3 as a Human Blood–Brain Barrier Model*. Molecular Pharmaceutics, 2013. **10**(1): p. 289-96.
174. Kumar, V. *et al.*, *Quantitative Transporter Proteomics by Liquid Chromatography with Tandem Mass Spectrometry: Addressing Methodologic Issues of Plasma Membrane Isolation and Expression-Activity Relationship*. Drug Metabolism and Disposition, 2015. **43**(2): p. 284-88.
175. Takano, M. *et al.*, *Interaction with P-glycoprotein and transport of erythromycin, midazolam and ketoconazole in Caco-2 cells*. European Journal of Pharmacology, 1998. **358**(3): p. 289-94.
176. Siebert, G. A., Hung, D. Y., Chang, P. & Roberts, M. S., *Ion-Trapping, Microsomal Binding, and Unbound Drug Distribution in*

the Hepatic Retention of Basic Drugs. Journal of Pharmacology and Experimental Therapeutics, 2004. **308**(1): p. 228.
177. Oda, K., Nishimura, Y., Ikehara, Y. & Kato, K., *Bafilomycin A1 inhibits the targeting of lysosomal acid hydrolases in cultured hepatocytes*. Biochemical and Biophysical Research Communications, 1991. **178**(1): p. 369-77.
178. Uchiyama, A. *et al.*, *Translocation of iron from lysosomes into mitochondria is a key event during oxidative stress-induced hepatocellular injury*. Hepatology, 2008. **48**(5): p. 1644-54.
179. Lazarska, K. E., Dekker, S. J., Vermeulen, N. P. E. & Commandeur, J. N. M., *Effect of UGT2B7*2 and CYP2C8*4 polymorphisms on diclofenac metabolism*. Toxicology Letters, 2018. **284**: p. 70-78.
180. Chen, R. & Snyder, M., *Systems biology: personalized medicine for the future?* Current Opinion in Pharmacology, 2012. **12**(5): p. 623-28.
181. Tavassoly, I., Goldfarb, J. & Iyengar, R., *Systems biology primer: the basic methods and approaches*. Essays in Biochemistry, 2018. **62**(4): p. 487-500.
182. Hughes, D. A. *et al.*, *Evaluating intra-and inter-individual variation in the human placental transcriptome*. Genome Biology, 2015. **16**(1): p. 54.
183. McCall, M. N., Illei, P. B. & Halushka, M. K., *Complex sources of variation in tissue expression data: analysis of the GTEx lung transcriptome*. The American Journal of Human Genetics, 2016. **99**(3): p. 624-35.
184. Ceriani, L. & Verme, P., *The origins of the Gini index: extracts from Variabilità e Mutabilità (1912) by Corrado Gini*. The Journal of Economic Inequality, 2012. **10**(3): p. 421-43.
185. Wang, D. *et al.*, *A deep proteome and transcriptome abundance atlas of 29 healthy human tissues*. Molecular systems biology, 2019. **15**(2): p. e8503.
186. Kmiec, Z., *Cooperation of liver cells in health and disease*. Advances in anatomy, embryology, and cell biology, 2001. **161**: p. Iii-xiii, 1-151.
187. Tortora, G. J. & Derrickson, B. H. *Principles of anatomy and physiology*. 12th edn, (John Wiley & Sons, 2008).
188. Lown, K. S. *et al.*, *Interpatient heterogeneity in expression of CYP3A4 and CYP3A5 in small bowel. Lack of prediction by the erythromycin breath test*. Drug Metabolism and Disposition, 1994. **22**(6): p. 947-55.
189. Hounnou, G., Destrieux, C., Desme, J., Bertrand, P. & Velut, S., *Anatomical study of the length of the human intestine*. Surgical and Radiologic Anatomy, 2002. **24**(5): p. 290-94.
190. Blomen, V. A. *et al.*, *Gene essentiality and synthetic lethality in haploid human cells*. Science, 2015. **350**(6264): p. 1092-96.

191. Ashburner, M. *et al.*, *Gene Ontology: tool for the unification of biology*. Nature Genetics, 2000. **25**(1): p. 25-29.
192. Gutiérrez-Sacristán, A. *et al.*, *DisGeNET: a comprehensive platform integrating information on human disease-associated genes and variants*. Nucleic Acids Res, 2016. **45**(D1): p. D833-D39.
193. Hjelmesæth, J. *et al.*, *Impact of body weight, low energy diet and gastric bypass on drug bioavailability, cardiovascular risk factors and metabolic biomarkers: protocol for an open, non-randomised, three-armed single centre study (COCKTAIL)*. BMJ Open, 2018. **8**(5): p. e021878.
194. Houten, S. M., Violante, S., Ventura, F. V. & Wanders, R. J. A., *The Biochemistry and Physiology of Mitochondrial Fatty Acid β-Oxidation and Its Genetic Disorders*. Annual Review of Physiology, 2016. **78**(1): p. 23-44.
195. Docanto, M. M., Ham, S., Corbould, A. & Brown, K. A., *Obesity-Associated Inflammatory Cytokines and Prostaglandin E2 Stimulate Glucose Transporter mRNA Expression and Glucose Uptake in Primary Human Adipose Stromal Cells*. Journal of Interferon & Cytokine Research, 2015. **35**(8): p. 600-05.
196. Gruchała-Niedoszytko, M. *et al.*, *Differences in gene expression related to the outcomes of obesity treatment, peak oxygen uptake, and fatty acid metabolism measured in a cardiopulmonary exercise test*. Polish archives of internal medicine, 2018. **128**(5): p. 280-86.
197. Sohn, J. H. *et al.*, *Perilipin 1 (Plin1) deficiency promotes inflammatory responses in lean adipose tissue through lipid dysregulation*. Journal of Biological Chemistry, 2018. **293**(36): p. 13974-88.
198. Cho, K. A. & Kang, P. B., *PLIN2 inhibits insulin-induced glucose uptake in myoblasts through the activation of the NLRP3 inflammasome*. International journal of molecular medicine, 2015. **36**(3): p. 839-44.
199. Wang, Q., Zheng, M. & Leil, T., *Investigating Transporter-Mediated Drug-Drug Interactions Using a Physiologically Based Pharmacokinetic Model of Rosuvastatin*. CPT: Pharmacometrics & Systems Pharmacology, 2017. **6**(4): p. 228-38.
200. Colburn, W. A., *Pharmacokinetic and biopharmaceutic parameters during enterohepatic circulation of drugs*. Journal of pharmaceutical sciences, 1982. **71**(1): p. 131-33.
201. Kameyama, Y., Yamashita, K., Kobayashi, K., Hosokawa, M. & Chiba, K., *Functional characterization of SLCO1B1 (OATP-C) variants, SLCO1B1*5, SLCO1B1*15 and SLCO1B1*15+C1007G, by using transient expression systems of HeLa and HEK293 cells*. Pharmacogenetics and Genomics, 2005. **15**(7): p. 513-22.

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