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Advanced In Vitro Systems for Studies of Drug Disposition in the Human Liver

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Abstract

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In drug development, *in vitro* models are used to assess specific aspects of *in vivo* Absorption, Distribution, Metabolism, Excretion and Toxicity (ADMET) properties of the drugs. Relevant *in vitro* assays play a crucial role in bringing safe and efficacious compounds to the market, and contribute to the Replacement, Refinement and Reduction (3Rs) of animal experiments.

Much effort is now being directed to the development of different physiologically relevant advanced *in vitro* models. One of such models is three-dimensional spheroids of primary human hepatocytes (3D PHH). These 3D PHH closely resemble the *in vivo* liver at the transcriptome, proteome and metabolome levels. However, 3D PHH are cultured under different conditions and the reproducibility of these culture varies greatly across laboratories. This thesis contributes to harmonization of 3D PHH culture approaches.

First, the effect of the cell culture medium on 3D PHH was evaluated. We compared various commercially available media with undisclosed or known content, and also assessed the influence of commonly used medium components such as glucose, insulin, zinc and foetal bovine serum. The choice of cell culture medium had a pronounced effect on the hepatic phenotypes. Importantly, we demonstrate that 3D PHH could be successfully cultured in the animal-serum free physiologically relevant medium with fasting levels of insulin and glucose.

Further, we appraised the effect of ultra-low attachment culture plates on the performance of 3D PHH, and demonstrated that Corning and Biofloat plates facilitate the formation of spheroids with most physiologically relevant phenotypes.

Throughout all projects included in this doctoral thesis, mass-spectrometry based global proteomics served as indispensable tool for phenotypic description of 3D PHH. However, the choice of workflow for this analysis has a significant impact on biological interpretation. Here, twelve different proteomics workflows for phenotypic description of 3D PHH were compared, and these results will aid researcher in our field in making an informed decision on the approach to the phenotypical screening of liver spheroid cultures.

In conclusion, this thesis provides an improved understanding and optimization of 3D primary human hepatocyte spheroid cultures, and deep integration of this *in vitro* model into drug development pipelines.

Keywords: Liver, In vitro models, Hepatocytes, PHH, Serum-free, Cell culture medium, Proteomics

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"Little snail
Ever so slowly, climb
Mount Fuji"

Kobayashi Issa

To my family and my friends

List of Papers

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

I. Handin, N., Mickols, E., Ölander, M., Rudfeldt, J., Blom, K., Nyberg, F., Senkowski, W., Urdzik, J., Maturi, V., Fryknäs, M., Artursson, P. (2021) Conditions for maintenance of hepatocyte differentiation and function in 3D cultures. *iScience,* 24(11):103235.

II. Mickols, E.[1], Meyer, A.[1], Handin, N.[1], Stüwe, M., Eriksson, J., Rudfeldt, J., Blom, K., Fryknäs, M., Sellin, M.E., Lauschke, V.M., Karlgren, M., Artursson, P. (2024) OCT1 (SLC22A1) transporter kinetics and regulation in primary human hepatocyte 3D spheroids. *Scientific Reports*, 14(1):17334.

III. Xing, C., Kemas, A., Mickols, E., Klein, K., Artursson, P., Lauschke, V.M. (2024) The choice of ultra-low attachment plates impacts primary human and primary canine hepatocyte spheroid formation, phenotypes, and function. *Biotechnology Journal,* 19(2):e2300587.

IV. Koutsilieri, S., Mickols, E., Végvári, Á., Lauschke, V.M. Proteomic workflows for deep phenotypic profiling of 3D organotypic liver models. (2024) *Biotechnology Journal,* 19(3):e2300684.

V. Mickols, E., Primpas, L., Oredsson, S., Karlgren, M. Animal product-free formation and cultivation of 3D primary hepatocyte spheroids. *(Submitted)*

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Abbreviations

3D PHH	3-dimensional spheroids of primary human hepatocytes
3Rs	Replacement, refinement and reduction of animal experiments
ABC	ATP binding cassette
ADMET	Absorption, distribution, metabolism, excretion and toxicity
ASP+	4-(4-(dimethylamino)styryl)-N-methylpyridinium
BCRP	Breast cancer resistance protein
BSEP	Bile salt export pump
CYP	Cytochrome P450
DDA	Data-dependent acquisition
DDI	Drug-drug interactions
DF	DMEM/Nutrient mixture F-12 (DMEM/F12)
DILI	Drug-induced liver injury
DMEM	Dulbecco's modified Eagle medium
DMEs	Drug metabolizing enzymes
DMSO	Dimethyl sulfoxide
DTT	Dithiothreitol
ECM	Extracellular matrix
ENT	Equilibrative nucleoside transporter
ER	Endoplasmic reticulum
FAIR	Findable, accessible, interoperable, and reusable
FBS	Foetal bovine serum
FMO	Flavin-containing monooxygenase
FT	Freeze-thaw
GO	Gene ontology
GST	Glutathione S-transferases
HBSS	Hank’s balanced salt solution
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HMM	Hepatocyte maintenance medium
HTS	High throughput screening
K_m	Michaelis–Menten constant
LC-MS/MS	Liquid chromatography - tandem mass-spectrometry
LF	Label-free
LFQ	Label-free quantification
MAO	Monoamine oxidases
MATE	Multidrug and toxin extrusion protein

MBR	Match-between-runs
MDR	Multidrug resistance
MED-FASP	Multiple enzymes digestion filter aided sample preparation
MRP	Multidrug resistance-associated protein
MS	Mass-spectrometry
NTCP	Sodium-taurocholate co-transporting polypeptide
OATP	Organic anion transporting
OCT1	Organic cation transporter
OSTα/β	Organic solute transporter alpha/beta
PCA	Principal components analysis
PEST	Penicillin-streptomycin
PHH	Primary human hepatocytes
PMU	ProteaseMAX/urea
PPM	Power primary HEP medium
PRIDE	Proteomics identification database
PTXQC	Proteomics quality control reports
RIPA	Radio immunoprecipitation assay
SDRF	Sample and data relationship format
SDS	Sodium dodecyl sulfate
SLC	Solute carrier
SP3	Solid-phase-enhanced sample-preparation
SULT	Sulfotransferase
TEAB	Triethylammonium bicarbonate
TMT	Tandem mass tag
TPA	Total protein approach
UGT	UDP-glucuronosyltransferase
ULA	Ultra-low attachment
V_{max}	Maximum velocity
Vsn	Variance stabilization normalization
WE	Williams´ E medium

Popular science summary (English)

Drug discovery and development is the process through which potential new drug candidates are identified and evaluated. An exceptional demand for safety and efficacy of the identified drugs makes this process rather long and very pricy.

There is a multitude of tools that could be used at the different stages of drug development. Some of them allow an investigation of the interaction between body organs and the drug in development. These tools are called *in vitro* (laboratory grown) cell cultures. These cultures could represent different organs, for example intestine, lung, skin and liver. The last plays a significant role in the drug development process.

The liver is a central hub for numerous physiological processes, namely metabolism of foodstuffs, synthesis of blood proteins, drug metabolism. etc. Drug metabolism is a process that aims to eliminate foreign substances from the body. In the liver, this task is assigned mostly to hepatocytes (main cell type in the liver). These cells take up the drug using designated transport proteins, break it down with the help of various enzymes, and excrete the modified compounds to be eliminated from the body. This process is also known as drug metabolism and disposition. It is fairly complex, and should be routinely assessed for each and every drug.

Obviously, these initial screening (also called preclinical) studies should not be performed in human volunteers or laboratory animals. Thus, *in vitro* cell cultures that mimic the liver come in handy. The emerging gold standard hepatic *in vitro* model is primary human hepatocytes spheroids (3D PHH). Essentially, it is a self-assembled microtissue (a tight ball of cells sized around one quarter of a millimetre) that consist of human hepatocytes isolated from human donor material — typically surgical waste from liver resection surgeries. These spheroids are formed by pipetting cell suspension in cell culture medium in specialised plates.

The aim of this doctoral thesis was to perform standardisation and functional benchmarking of 3D PHH spheroid cultures as a reliable *in vitro* model for drug development.

In Paper I, we characterised the variability observed in liver spheroid culture conditions. Specifically, we pointed out that these cultures could be kept in different cell culture media and that the composition of the media has a significant influence of the performance of liver cells. One of the main

findings in this paper is that 3D PHH could be cultured in physiologically normal medium. Traditionally, cell cultures are kept in a medium with very high insulin (thousand times higher than in the human blood) and excessive glucose (diabetic levels). That is done to promote efficient growth of cells in a laboratory environment. In Paper I, we found that these conditions are not necessary for 3D PHH cultures, and that spheroids perform equally as good in the medium with glucose and insulin close to fasting blood levels.

In Paper II, we cultured liver spheroids in this physiological medium and performed drug-drug interaction studies. These studies are often included in the drug development pipelines, as patients typically take multiple drugs at the same time, and these drugs could affect each other and cause unwanted side effects. Food and beverages could also cause this effect. In Paper II we demonstrated that liver spheroids in the physiological medium could be successfully used for these studies in both short- and long-term settings.

In Paper III, we proceeded with optimisation of liver spheroid cultures and evaluated the performance of the aforementioned specialised ultra-low attachment plates that are used for formation and maintenance of spheroids.

In Papers I-III we used global proteomics analysis to evaluate the phenotypes of liver spheroids. This type of analysis allows to measure several thousand proteins at the same time and by this, draw a biological hypothesis on the performance of the studied cells. In Paper IV, we appraised twelve different proteomics analysis workflows and how the choice of proteomics workflow could affect the biological conclusion that is made in the analysis.

Lastly, in Paper V we further developed 3D PHH cultures. As we demonstrated in Paper I, the composition of cell culture medium has a pronounced impact on the spheroids. Importantly, cell culture medium is often supplemented with foetal bovine serum (a product derived from the blood of unborn calves). This additive is an exceptionally rich mixture of proteins and nutrients, and for the last seventy years has been used to culture cells in the laboratory settings. However, the composition of foetal bovine serum varies largely between batches, which affect the reliability of the experiments performed in the presence of this additive. In addition, serum production itself raises ethical concerns. In Paper V we demonstrate that for liver spheroid cultures foetal bovine serum could successfully be substituted with a cocktail of proteins, hormones and nutrients that are either derived from the human body or chemically synthesised.

In summary, this doctoral thesis contributes to the standardisation of 3D PHH cultures. Here we evaluate the approaches that are typically used to maintain and analyse liver spheroid cultures, and expand the realm of cell culture conditions that could successfully be used to culture 3D PHH for a variety of experiments. For instance, physiologically relevant medium could be used in metabolic dysfunction-associated steatohepatitis studies, allowing to assess the broad spectrum of physiological variability in the human liver, nutritional intake and metabolic makeup.

Populärvetenskaplig sammanfattning (Swedish)

Läkemedelsutveckling är den process genom vilken potentiellt nya läkemedel identifieras. Det ställs höga krav på läkemedels effekt och säkerhet vilket gör läkemedelsutveckling till en komplicerad och kostsam process.

Många olika metodiker används under läkemedelsutvecklingens olika stadier från provrörsexperiment till studier i människa. Några av dem används för att ge en tidig bild av läkemedlets öde i våra kroppar, alltså vad kroppen gör med läkemedlet. En av dessa metoder är cellodlingar som används för att förutsäga hur läkemedel tas upp, utövar sin effekt, bryts ned och utsöndras i respektive organ. Cellodlingar baserade på leverceller är bland de viktigaste under i läkemedelsutvecklingsprocessen.

Levern är central för många fysiologiska processer, som till exempel tillverkning av blodproteiner och nedbrytning av livsmedel. Även läkemedel bryts ner i levern. Detta sker i leverceller som kallas hepatocyter. Läkemedel transporteras in i cellerna med hjälp transportproteiner. Därefter bryts läkemedlet ner med hjälp av olika enzymer och sedan utsöndras restprodukterna från cellen och från kroppen. Det här är en komplicerad process som rutinmässigt måste utredas för varje potentiellt läkemedel.

Dessa initiala undersökningar i läkemedelsutveckling bör självfallet inte utföras på människor eller laboratoriedjur. Istället använder man cellkulturer som liknar mänsklig lever. Den cellkultur som just nu är mest lovande är hepatocytsfäroider av celler isolerade från mänsklig levervävnad – oftast vävnad som blivit över vid kirurgiska ingrepp. De här sfäroiderna är små kompakta bollar av leverceller med en storlek av ca fjärdedels mm som bildas genom att man tillsätter en viss mängd celler i speciella cellodlingsplattor där cellerna sedan kan fästa till varandra.

Syftet med den här doktorsavhandlingen var att undersöka hur man på ett standardiserat sätt kan ta fram och använda de här sfäroiderna för att få så tillförlitliga resultat som möjligt under läkemedelsutvecklings-processen.

I Artikel I undersökte vi hur sfäroiderna påverkas av olika odlingsförhållanden. Framförallt undersökte vi hur olika cellodlingsmedium (näringslösning som sfäroiderna odlas i) påverkade sfäroidernas funktion. Ett huvudfynd i artikeln är att sfäroiderna kunde odlas i ett medium som liknar miljön som levercellerna exponeras för i människa. Traditionellt odlas cellkulturer i ett medium med mycket höga koncentrationer av insulin (tusentals gånger högre

än vad som finns i blodet) och höga sockerhalter (liknande nivåer som vid diabetes). Detta görs för att cellerna ska växa bra i en laboratoriemiljö. I vår studie såg vi att detta inte är nödvändigt för sfäroidkulturerna utan att de fungerar lika bra i mediet som har samma socker- och insulinhalter som i vårt blod har vid fasta.

I Artikel II odlade vi sfäroiderna i det fysiologiska mediet vi tog fram i Artikel I och undersökte interaktioner mellan olika läkemedel. Sådana studier är ofta inkluderade i läkemedelsutvecklingsprocessen. Detta eftersom det är vanligt att patienter tar flera läkemedel samtidigt, och då läkemedlen kan påverka varandra (interaktioner) och orsaka biverkningar. Även viss mat och dryck kan orsaka interaktioner. I Artikel II visade vi att sfäroider odlade i det fysiologiska mediet framgångsrikt kunde användas för den här typen av interaktionsstudier.

I Artikel III fortsatte vi optimera sfäroidkulturerna och det vi då undersökte var hur specialplattorna som man odlar cellerna i för att de ska forma sfäroider påverkade kulturerna.

I Artikel I-III använde vi oss av en analysmetod där man kan mäta flera tusen proteiner samtidigt för att undersöka hur cellerna mår och fungerar. I Artikel IV undersökte vi tolv olika protokoll för att göra sådana analyser och hur valet av protokoll kan påverka resultaten vi får och därmed vilka slutsatser vi drar.

Till sist, i Artikel V fortsatte vi att vidareutveckla sfäroidkulturerna. Som vi visade i Artikel I påverkar cellodlingsmediumet hur sfäroiderna fungerar. En vanlig tillsats i cellodlingsmedium är serum som man får från blodet från kalvfoster. Den här tillsatsen innehåller mycket proteiner och näringsämnen och har under de senaste sjuttio åren använts för att odla celler i en laboratoriemiljö. Sammansättningen av kalvserum varierar dock mycket vilket påverkar tillförlitligheten av experimenten. Dessutom finns det etiska problem med själva produktionen av serum. I Artikel V visar vi att vid odling av sfäroider kan kalvserum ersättas med en mer standardiserad cocktail av proteiner, hormoner och näringsämnen som antingen är renade från mänsklig vävnad eller syntetiskt framställda.

Sammanfattningsvis bidrar den här avhandling till information om hur vi på ett standardiserat och reproducerbart sätt kan använda hepatocytsfäroider under läkemedelsutvecklingsprocessen. Vi utvärderar här olika analysprotokoll och odlingsmetoder för att på så sätt skapa förutsättningar för olika typer av experiment. Till exempel kan det fysiologiskt relevanta mediet vi utvecklade göra det lättare att undersöka fysiologiska och funktionella skillnader hos levern vid olika leversjukdomar

Научно-популярный реферат-резюме докторской монографии (Russian)

Разработка лекарств — это сложный процесс, посредством которого идентифицируются потенциально новые лекарственные вещества. Исключительное требование к безопасности и эффективности идентифицированных молекул превращает процесс разработки лекарственных средств в длительный и дорогостоящий.

В настоящее время ученым доступно множество моделей, которые можно использовать на разных этапах разработки лекарств. Некоторые из них позволяют исследовать реакцию органов на воздействие разрабатываемых препаратов. Одной из таких моделей являются *in vitro* клеточные культуры, которые могут представлять различные органы, например, кишечник, легкие, кожу и печень.

Печень является центральным узлом для многочисленных физиологических процессов, например: переработки пищевых продуктов, синтеза белков крови и метаболизма лекарств, который в свою очередь направлен на выведение чужеродного вещества из организма. Эти задачи, в основном, возложены на гепатоциты (основной тип клеток в печени), которые поглощают лекарство с помощью определенных транспортных белков, расщепляют его с помощью различных ферментов и выделяют измененные соединения из организма. Этот сложный и многокомпонентный процесс называется диспозицией лекарственных средств. Детальное изучение этого процесса является основой понимания профиля безопасности разрабатываемого препарата, и фармацевтические компании обязаны детально оценивать этот процесс.

Очевидно, что такие первоначальные скрининговые исследования не могут быть проведены на добровольцах или лабораторных животных. Таким образом, *in vitro* культуры печени являются незаменимым инструментом в разработке лекарственных средств. Новый золотой стандарт клеточных *in vitro* моделей печени — это сфероиды человеческих гепатоцитов. По существу, это самоорганизованная микроткань (размером около четверти миллиметра), которая состоит из гепатоцитов, выделенных из донорского материала человека, как правило, хирургических отходов от операций по резекции печени. Эти

сфероиды формируются в результате пипетирования клеточной суспензии в культуральной среде в специализированный пластик.

Целью докторской диссертации было проведение стандартизации и функционального сравнительного анализа сфероидов гепатоцитов и оценка этой клеточной культуры, как надежной *in vitro* модели для разработки лекарственных средств.

В Статье I мы охарактеризовали изменчивость, наблюдаемую в условиях культивирования сфероидов гепатоцитов. В частности, мы указали, что эти культуры можно содержать в различных средах, и состав среды оказывает значительное влияние на показатели печеночных функций клеток. Одним из основных выводов в этой работе является то, что сфероиды гепатоцитов можно культивировать в физиологически нормальной среде. Традиционно клеточные культуры содержатся в среде с очень высоким содержанием инсулина (в тысячи раз выше, чем в крови человека) и избыточной глюкозой (диабетические уровни). Это, как правило, способствует эффективному росту клеток в лабораторных условиях. Однако, в процессе исследования нами было определено, что сфероиды гепатоцитов замечательно функционируют в среде с глюкозой и инсулином, близкими к уровням крови натощак.

В Статье II мы культивировали сфероиды печени в этой физиологической среде и провели исследования взаимодействия лекарственных средств, результаты которых всегда включаются в разработку лекарств, поскольку пациенты обычно принимают несколько препаратов одновременно. Препараты могут влиять друг на друга и вызывать нежелательные явления (еда и напитки обладают аналогичным действием). В своей Статье II мы продемонстрировали, что сфероиды печени в физиологической среде могут успешно использоваться для этих исследований как в краткосрочной, так и в долгосрочной перспективе.

В Статье III мы продолжили оптимизацию культур сфероидов печени и оценили влияние вышеупомянутого специализированного пластика, который используются для формирования и поддержания сфероидов.

В Статьях I-III мы использовали глобальный анализ протеома (совокупность всех белков клетки) для оценки фенотипов сфероидов печени. Этот тип анализа позволяет измерять несколько тысяч белков одновременно, и тем самым выдвигать биологическую гипотезу о производительности изучаемых клеток. В Статье IV мы оценили двенадцать различных подходов к анализу протеома и то, как выбор рабочего процесса протеомики может повлиять на биологический вывод, сделанный в ходе анализа.

Наконец, в Статье V мы продолжили разработку культур сфероидов печени. Как мы продемонстрировали в работе I, состав среды для культивирования клеток оказывает выраженное влияние на сфероиды. Важно отметить, что среда для культивирования клеток часто

дополняется сывороткой плода крупного рогатого скота (продуктом, полученным из крови нерожденных телят). Эта добавка представляет собой исключительно богатую смесь белков и питательных веществ и в течение последних семидесяти лет используется для культивирования клеток в лабораторных условиях. Однако, состав сыворотки плода крупного рогатого скота в значительной степени различается между партиями, что влияет на надежность экспериментов, проводимых в присутствии этой добавки. Кроме того, само производство сыворотки вызывает этические вопросы. В Статье V мы демонстрируем, что для культур сфероидов печени сыворотку плода крупного рогатого скота можно успешно заменить коктейлем из белков, гормонов и питательных веществ, которые либо получены из человеческого организма, либо синтезированы химическим путем.

Подводя итог, эта докторская диссертация вносит значимый вклад в стандартизацию культур сфероидов печени. Здесь мы оцениваем подходы, которые обычно используются для поддержания и анализа сфероидов печени, и расширяем область условий культивирования клеток, которые могут быть успешно использованы для различных экспериментов. Например, физиологически релевантная среда может быть использована в исследованиях стеатогепатита, связанного с метаболической дисфункцией, что позволяет оценить широкий спектр физиологической изменчивости в печени человека. Стоит отметить, что подход к разработке и улучшению клеточной среды, представленный в этой докторской диссертации, с легкостью может быть использован для других клеточных *in vitro* культур. В частности, мы провели пилотные исследования использования разработанной нами физиологической среды на органоидах тонкого кишечника человека и получили многообещающие результаты. Кроме того, эксперименты по замене сыворотки плода крупного рогатого скота (описанные в Статье V) были успешно повторены нами на других клеточных линиях, используемых в разработке лекарств, например на клеточной линии аденокарциономы человека Caco-2. Таким образом, результаты, показанные в данной диссертации вносят вклад не только в характеризацию и разработку сфероидов печени, но и гармонизацию и усовершенствование всех клеточных *in vitro* моделей, используемых в процессе разработки лекарственных средств.

Introduction

Drug development is a risky endeavour. An exceptional demand for drug safety and efficacy makes this process timely and costly. Hence, predicting how the leading compound will behave in humans as early as possible is essential. Currently, the industry is making a major leap in substituting traditional *in vivo* animal-based with *in vitro* assays, which can act as reliable surrogates for specific aspects of *in vivo* Absorption, Distribution, Metabolism, Excretion and Toxicity (ADMET) properties of the drugs.[1,2] Relevant *in vitro* assays play a crucial role in bringing safe and efficacious compounds to the market. Additionally, effective *in vitro* assays contribute to the Replacement, Refinement and Reduction (3Rs) of animal experiments.[3,4]

Many drugs are substrates for hepatic transporters and drug metabolizing enzymes (DMEs), which can alter compound ADMET properties.[5] These effects are have to be investigated during development of a new drug. Moreover, the liver is highly susceptible to drug toxicity, and drug-induced liver injury (DILI) account for the majority of acute liver failure cases in clinic.[6] DILI is one of the main reasons for the termination of clinical trials or compound withdrawal from the market.[7,8] Various *in vitro* hepatic models are used to estimate drug ADMET properties.[9–13] One of the most sophisticated and physiologically relevant *in vitro* liver models is 3D spheroids of primary human hepatocytes (herein and after referred to as 3D PHH). This *in vitro* model is currently getting increasingly integrated into drug development pipelines.[8,14,15]

However, the use of 3D PHH is not straightforward. First, primary human hepatocytes do not proliferate *in vitro* under normal conditions, and, thus, researchers rely on the limited availability of cryopreserved hepatocytes.[16] Further, cryopreserved hepatocyte donor batches vary broadly in quality and ADMET-related properties such as drug transporter' and enzyme' expression and function.[17,18] For *in vitro* ADMET assays, a comprehensive description of the hepatic cellular phenotype and culture conditions is paramount for the correct interpretation of experimental results. Moreover, the potential harmonisation of these parameters shall aid the comparability of the research results obtained in different laboratories. This thesis aims to improve the current understanding of 3D PHH *in vitro* model for studying drug disposition in the human liver. In addition to 3D PHH characterisation; improved and physiologically relevant cell culture conditions are developed, fully characterized, and tested in drug disposition experiments.

The human liver

Liver functions

The liver is the largest internal organ in the human body, employing around 2% to 5% of the total body weight and nearly a quarter of the total cardiac output via the hepatic artery and the portal vein. [19,20] The liver is a central hub for numerous physiological processes: macronutrient metabolism, immune system support, lipid and cholesterol homeostasis, breakdown of xenobiotic compounds, and many more (Figure 1). [20,21]

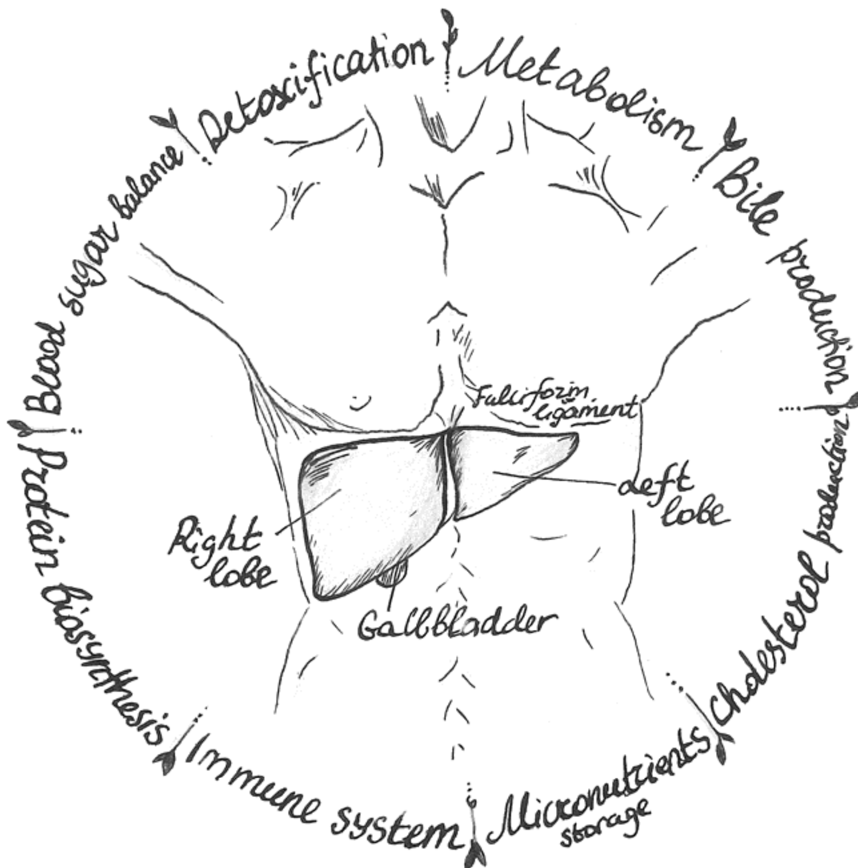

Figure 1. Summary of liver functions. The liver performs multiple physiological processes: macronutrient metabolism, immune system support, lipid and cholesterol homeostasis, breakdown of xenobiotic compounds, and many more.

The importance of the human liver could not be understated — proper functioning of this organ is essential to life, and liver failure typically necessitates complex transplantation procedure. The architecture of this organ (described below) ensures the polarisation and functional specialisation of the cells, that

permanently ensure homeostasis of the whole body. Typically, liver functions are categorised into several overarching categories.

First, the liver is a pivot of metabolism.[21] This encompasses carbohydrates metabolism, which includes storing glucose in the form of glycogen or releasing glucose to the blood by breaking down glycogen or *de novo* glucose synthesis (gluconeogenesis); protein metabolism, involving synthesis and degradation of plasma proteins and coagulation factors and supply of amino acids to the blood; lipid metabolism, namely cholesterol synthesis, lipogenesis, production of lipoproteins and production of bile. Bile ensures the absorption of dietary fat and lipophilic vitamins. Further, the liver is responsible for the detoxification of endo- and xenobiotics that could potentially harm the body. These compounds include endogenous molecules like bile salts and bilirubin, and exogenous molecules like drugs and toxins.

Tightly connected to the metabolic function, bile production of the liver ensures the uptake of desired foodstuffs like dietary lipids and fat-soluble vitamins, and excretion of the many solutes that are not excreted by the kidney. The latter include cholesterol, bile pigments, trace minerals, plant sterols, lipophilic drugs and metabolites, and other compounds that do not pass the glomerular filtration, often because these compounds are bound to the serum albumin. Of note, metabolism and bile production are performed in hepatocytes.

Additionally, liver serves as a storage of vitamins A, D, E, K, B12 and metals such as iron, zinc, and cobalt. It is also responsible for the purification of blood from bacterial pathogens, a process performed by phagocytic Kupffer cells.

Partially due to the multidimensional functionality, the liver is prone to many maladies, for instance alcoholic and non-alcoholic fatty liver diseases largely caused by excessive alcohol intake or unbalanced diet.[22] Another example is hereditary liver disorders like Wilson's disease — loss of copper-transporting ATPase 2 that typically excretes excessive copper in the bile — that results in accumulation of copper in the body and multiorgan failure.[23]

Functional anatomy of the liver

To perform these many roles, the liver is strategically placed in the circulatory system to receive blood from the gastro-intestinal tract (with exception for oesophagus and colon), pancreas and spleen.[21]

Cells in the liver are organised in thousands of small functional units called lobules (Figure 2). Lobules comprise hepatocyte cords organised in a hexagonal shape around the central vein. At the ventricles of each hexagon, a portal triad of the hepatic artery, portal vein, and bile duct is placed.

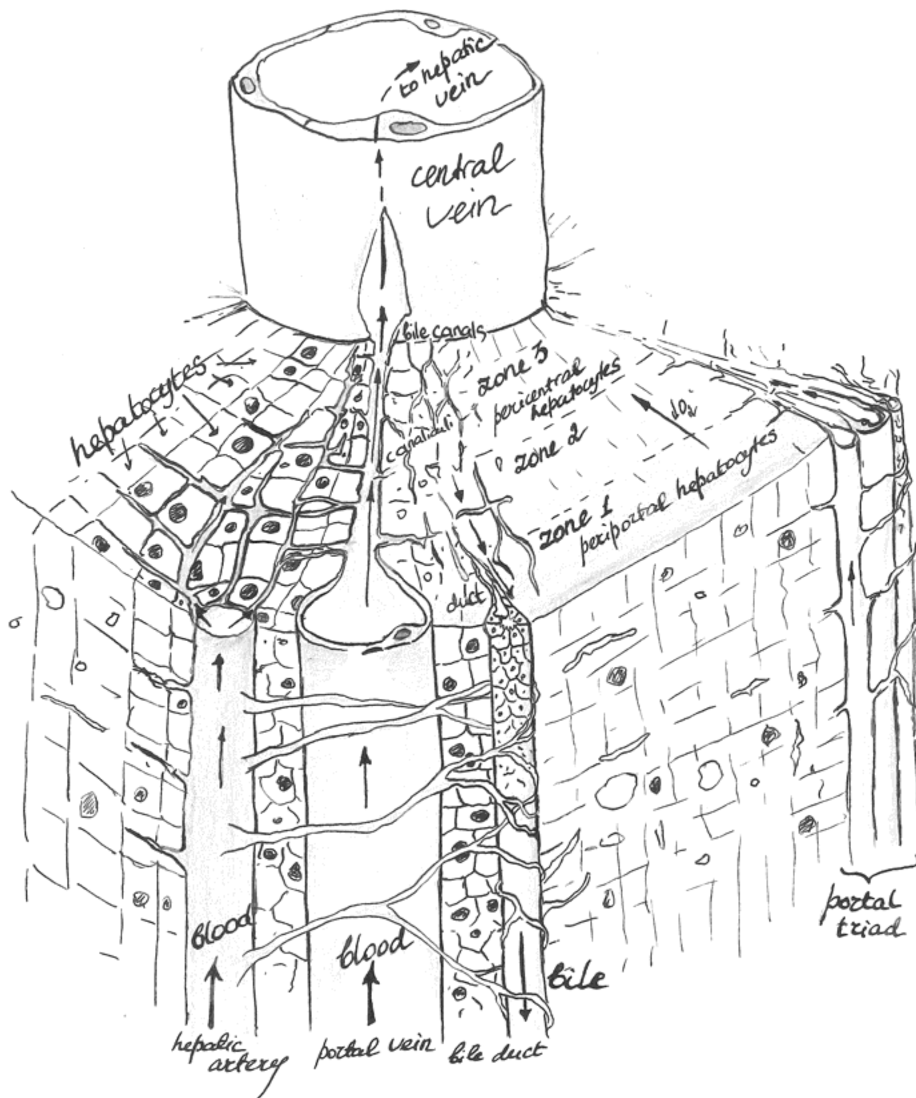

Figure 2. Microstructure of the liver. The liver is divided into structural hexagonal units called lobules, where hepatocytes form a sinusoidal network to facilitate the handling endo- and xenobiotics.

This architecture facilitates the formation of blood-enriched hepatic cell networks: nutrient-rich blood from the portal vein (that collects products of digested food) mixes with the oxygen-rich blood from the hepatic artery and flows through the lobule sinusoids to the central vein. This peculiar organization establishes a graded microenvironment of oxygen, nutrients and metabolic by-products in the hepatocyte cords, resulting in the phenotypic and functional partitioning of the hepatocytes known as “liver zonation” (Figure 2).[24]

The liver is comprised of several distinct cell types, including hepatocytes, cholangiocytes, stellate cells, Kupffer cells, and liver sinusoidal endothelial cells (Figure 3).[25,26] Each cell type possesses a unique set of functions that cooperatively contributes to the performance of the whole organ. For instance, cholangiocytes line the lumen of the bile ducts, while stellate cells store vitamin A in lipid droplets.[21,26] Kupffer cells are resident liver macrophages that are the first cells in the liver that come in contact with the microbial debris derived from the gastrointestinal tract.[27] Lastly, liver sinusoidal endothelial cells are highly specialized endothelial cells that form a highly penetrable fenestrated barrier that facilitates the permeation of compounds to hepatocytes.

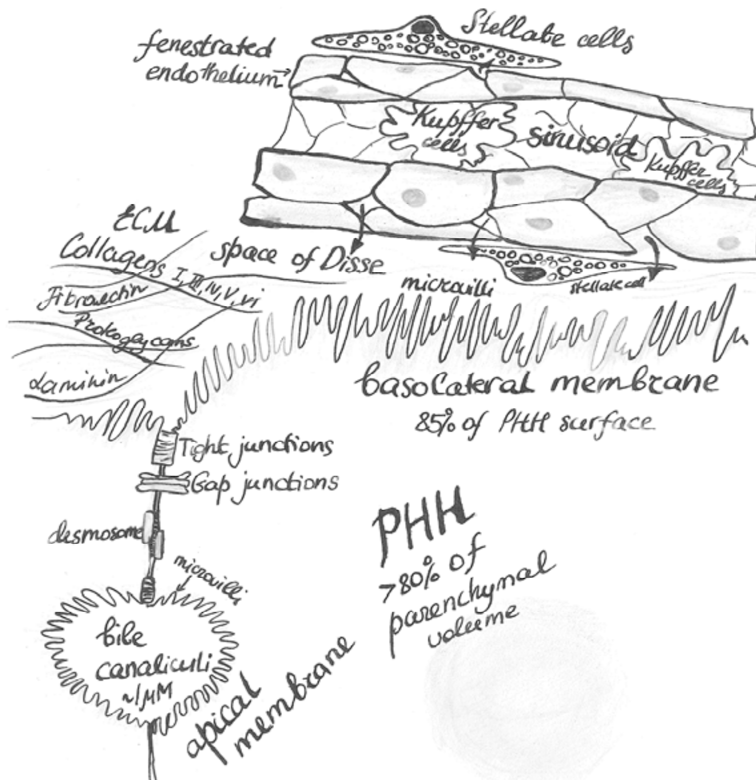

*Figure 3.*The liver contains highly specialised and polarised cells to perform the required organ functions.

Hepatocytes, that are in focus of this doctoral thesis, constitute the primary epithelial cells population in the liver and comprise around 80% of the organ volume. [20] Notably, hepatocytes are responsible for the metabolism and detoxification of many endogenous and exogenous compounds. In the lobule, hepatocytes form an epithelium that functions as a barrier between sinusoidal space that contains blood and canalicular lumen filled with bile (Figures 2 and 3). This architecture co-occurs with the formation of highly polarised hepatic

epithelium, where the apical membrane (facing canalicular volume) and basal membrane (sinusoid-facing) differ distinctly in their function and expression of transport proteins. [28] Basolateral membranes are typically enriched in microvilli that project into the space of Disse to facilitate contact with the solutes in blood; while apical membranes form a narrow belt sealed by tight junctions that form a barrier to the excreted bile in the canalicular space (Figure 3). Of note, this polarity is quintessential for the maintenance of appropriate endo- and xenobiotic handling and transportation.

The described architecture relies on the scaffold of extracellular matrix proteins (ECM) like collagens (I, III, IV, V, and VI), fibronectin and laminin. [21] Of note, ECM not only provides essential structural support, but also controls cell behaviour by providing adhesion and immunomodulatory signals, as well as by acting as a reservoir of growth factors and cytokines.[29–31]

Drug disposition in the liver

It is well recognised that the liver is the main metabolism site for many drugs. Once a compound is delivered to the liver, it is typically distributed into hepatocytes to be transformed and eliminated.

This process starts (and also later on ends) with a drug passing through the cellular membrane either by passive diffusion according to the chemical gradient or via carrier-mediated transport.[32] While passive diffusion depends mostly on the molecular properties of the drug, carrier-mediated transport relies on membrane transporter proteins, divided into two major superfamilies — the solute carrier (SLCs) superfamily and the ATP binding cassette (ABCs) superfamily.[32,33] These proteins are called active transporters since they typically require energy in form of ATP hydrolysis or ion gradients.[34]

Although these transporter superfamilies contain numerous proteins expressed in human hepatocytes, only a subset of these (Figure 4) is relevant for drug transporting and, in particular, for drug-drug interaction studies (DDIs), non-response reactions and drug toxicity.[33] Of note, localisation of these transporters in the hepatocytes is highly specialised, e.g. OATPs and OCT1 are present only on the basolateral membrane facilitating the uptake from blood, while MDR1 and BSEP only on the apical membrane, excreting to bile.

Once a drug is inside hepatocytes, metabolising enzymes join in labour to convert the xenobiotic into a more hydrophilic derivative to facilitate the elimination of the compound out of the body (Figure 4 and Table 1).[35] This process is traditionally divided into two major phases: phase I reactions that represent oxidation or reduction largely catalysed by cytochromes P450 (CYPs), but also monoamine oxidases (MAOs) and flavin-containing monooxygenase (FMO); and phase II reactions of conjugation to a highly hydrophilic compound such as glucuronate (by UDP-glucuronosyltransferases, UGTs), sulfate

(by sulfotransferases, SULTs), or glutathione (glutathione S-transferases, GSTs).[21]

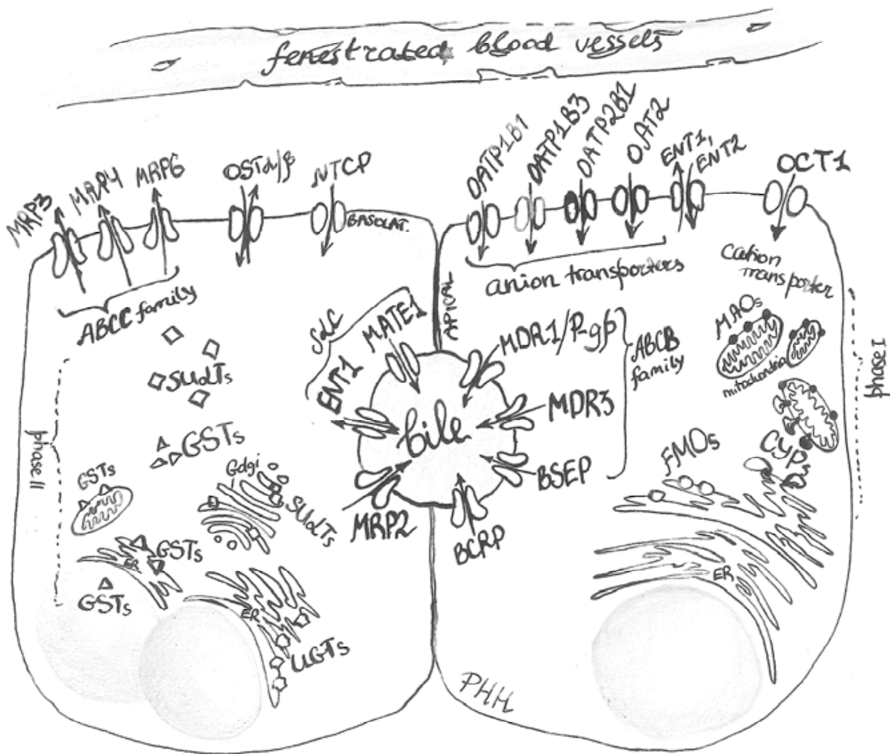

Figure 4. Localisation of important drug disposition transporters and drug metabolising enzyme families. [33,36–48] Basolateral membrane transporters: MRP3,4,6, multidrug resistance-associated protein (*ABCC3,4,6*); OSTα/β, organic solute transporter alpha/beta (*SLC51A/B*); NTCP, sodium-taurocholate co-transporting polypeptide (*SLC10A1*); OATP1B1,3, organic anion transporting polypeptide (*SLCO1B1,3*); OATP2B1, organic anion transporting polypeptide 2B1 (*SLCO2B1*); ENT1,2, equilibrative nucleoside transporter 1 (*SLC29A1,2*); OCT1, organic cation transporter (*SLC22A1*). Apical membrane transporters: BCRP, breast cancer resistance protein (*ABCG2*); BSEP, bile salt export pump (*ABCB11*); MDR3, multidrug resistance protein 3 (*ABCB4*); MDR1/P-gp, P-glycoprotein (*ABCB1*); MRP2, multidrug resistance-associated protein (*ABCC2*); ENT1; MATE1, multidrug and toxin extrusion protein (*SLC47A1*). Phase I drug metabolising enzymes: cytochromes P450 (CYPs); monoamine oxidases (MAOs); flavin-containing monooxygenase (FMO). Phase II drug metabolising enzymes: uridine 5'-diphospho-glucuronosyltransferases (UDP-glucuronosyltransferases, UGTs); glutathione S-transferases (GSTs); sulfotransferases (SULTs).

Of note, despite the naming, phase I and II metabolism does not necessarily occur one after the other. The drug could undergo biotransformation by the listed enzymes subsequently, partially, simultaneously or not at all. This redundancy in the detoxification system ensures efficient and rapid removal of

a variety of xenobiotics. Typically, a mixture of the native xenobiotic and phase I and II metabolites is excreted by the aforementioned transporters to the bile or systemic circulation for further excretion. The excretion of metabolised compounds is sometimes referred to as Phase III.

Table 1. Important drug metabolising enzymes presented in human hepatocytes.

CYPs[49,50]	**MAOs**[51]	**FMOs**[52]	**UGTs**[53]	**GSTs**[54]	**SULTs**[55–57]
	Phase I			*Phase II*	
CYP1A2	MAO-A	FMO3	UGT1A1	GSTA1	SULT1A1
CYP2A6	MAO-B	FMO4	UGT1A3	GSTA2	SULT1A3
CYP2B6		FMO5	UGT1A6	GSTA4	SULT1B1
CYP2C8			UGT1A8	GSTM1	SULT1C2
CYP2C9			UGT1A10	GSTM3	SULT1E1
CYP2C19			UGT2B4	GSTM4	SULT2A1
CYP2D6			UGT2B7	GSTP1	
CYP2J2				GSTT1	
CYP3A4				GSTT2	
CYP3A5					

Importantly, even though the main aim of liver drug metabolism is the metabolic deactivation of xenobiotics, some of the drugs become reactive only after the biotransformation by liver enzymes. For instance, commonly prescribed antipyretic paracetamol (also known as acetaminophen) is metabolised by CYP2E1 to reactive paracetamol metabolite, acetyl-p-benzoquinone imine (NAPQI), which facilitates the formation of free radicals and hepatocyte damage.[58] This reaction is a classic example of metabolic transformation leading to drug-induced liver injury (DILI). [8,59]

Furthermore, many xenobiotics are ligands for nuclear transcription factors, also termed as xenobiotic receptors, namely aryl hydrocarbon receptor (AhR), constitutive androstane receptor (CAR), pregnane X receptor (PXR), peroxisome proliferator-activated receptor α (PPARα), nuclear factor erythroid 2-related factor 2 (Nrf2), farnesoid X receptor (FXR), and liver X receptor (LXR). [60,61] Upon xenobiotic binding these nuclear receptors collaboratively alter the transcription of a broad range of DMEs, as well as uptake and efflux transporters.

To sum up, hepatic drug disposition is an intricate interplay between transporters, enzymes, nuclear receptors and other parameters that could affect the performance of these factors, namely cellular health state, genetic factors, exposure to nutrients and cofactors, etc. To correctly estimate drug disposition during the drug development process, these factors ought to be assessed. For this purpose, physiologically relevant *in vitro* hepatic models are essential.

In vitro primary human hepatocyte models

A plethora of *in vitro* human hepatic models could be used to study drug disposition in the liver.[62–69] In brief, these models vary in cellular sources, e.g. human hepatoma cell lines, induced pluripotent stem cell (iPSC) or primary human hepatocytes (PHH), and architectural complexity: monolayers, organoids, liver-on-a-chip platforms. This ultimately results in significant variability in molecular phenotype and functional applications between *in vitro* hepatic models. Each cell model has benefits and limitations, which should be considered when designing relevant *in vitro* assays.

The gold standard cell source for human hepatic *in vitro* models is primary human hepatocytes (PHH). Primary human hepatocyte transcriptomes and proteomes are closely resembling the ones of the patient liver.[67,70,71] Moreover, PHH retain donor-specific phenotypes, thus *in vitro* studies performed in a sufficient variety of PHH donor batches could emulate the pharmacological profile of the human population. Additionally, PHH are metabolically active, and are used to determine the drug candidates' metabolic clearance.[15] Lastly, PHH show the highest sensitivity in hepatic toxicity studies and are successfully used to predict DILI.[8,14] The main drawback associated with the use of PHH is their limited availability due to the inability to spontaneously divide *in vitro* under regular conditions.[72,73] PHH could be cultured in multiple configurations (Figure 5).

PHH suspension (Figure 5a) is a well-established short-term model to determine metabolic clearance of drug compounds.[74–76] PHH suspensions are best suited for shorter incubation times (4-6 hours) due to the inherent decrease in viability and metabolic activity over time. These constraints limit the applicability of PHH suspensions in long-term clearance and toxicity studies. The relay method was proposed to circumvent these limitations.[77] This method involves multiple transferring the supernatant from hepatocyte incubations to freshly thawed hepatocytes at the end of 4-h incubation periods.

PHH monolayers seeded on rat tail collagen I (Figure 5b) is another widely used hepatic system for mimicking the human liver in drug transport, metabolism and toxicity studies.[17,78,79] Monolayer cultures are straightforward to handle, yet when seeded in 2D format, PHH rapidly undergo epithelial-mesenchymal transition (EMT) and rapidly dedifferentiate, loosing metabolic capacity within 48 hours.[12,80] PHH are terminally differentiated cells and are highly dependent on the spatiotemporal regulation by microenvironmental signals in liver lobules — cues that are often lost in a 2D setting. To circumvent this issue, a chemically-defined approach was established to extend the culture time of viable and metabolically competent PHH monolayers for up to one month.[81,82] This method encompasses the incubation of PHH with a cocktail of chemicals and small-molecule inhibitors of EMT.

Alternatively, sandwich PHH culture (Figure 5c) could be used. PHH in this format are suitable for intermediate-term drug metabolism and transport

studies for up to several weeks.[83] In PHH sandwich culture, hepatocytes are overlaid with the additional coating of extracellular matrix to mimic spatial organization of the liver and prompt correct apical-basolateral polarity and formation of the bile canaliculi. However, long-term sandwich cultures demonstrated bile canaliculi damage and cholestasis development due to the absence of the bile outflow from the formed canaliculi.[84,85]

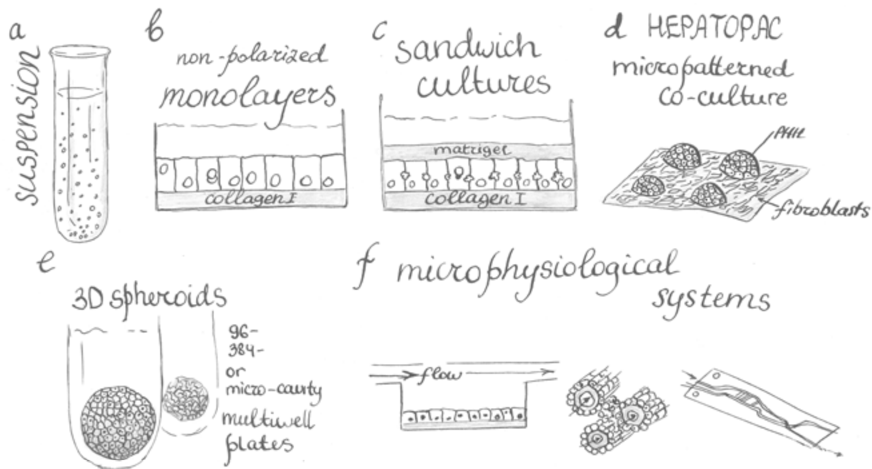

Figure 5. Examples of culture configurations of primary human hepatocytes. a. Short-term hepatocyte suspension. b. Conventional 2D monolayer — hepatocytes are seeded on the surface coated with an extracellular matrix, typically rat-tail collagen I. c. Sandwich configuration — hepatocytes are overlaid with an additional layer of extracellular matrix, typically Matrigel. d. Micropatterned co-culture — hepatocyte "islands" are seeded between fibroblast layer. e. 3D spheroid culture — PHH aggregate when seeded on ultralow attachment (ULA) surfaces. f. Microfluidic devices are designed to mimic liver biophysical factors, including hemodynamic, shear stress and lobule architecture.

Further, micropatterned coculture (MPCC) systems were developed to maintain PHH differentiation (Figure 5d). The most commonly used MPCC is the commercially available HEPATOPAC® system, comprised of hepatocyte "islands" surrounded with supportive stromal cells.[86–88] This architecture promotes differentiated hepatic cultures for at least 28 days.

Also, three-dimensional (3D) PHH spheroid (Figure 5e) cultures are emerging as promising hepatic models that promote hepatocyte phenotype and functionality for longer time in culture.[12,14,15,89,90] 3D PHH spheroids are used for drug metabolism, long-term toxicity studies, and liver biology investigations. There are multiple ways to generate these spheroids. For instance, stirring bioreactors, aggregation in hanging drops, or culture on ultralow attachment (ULA) surfaces.[89] The latter is certainly the most straightforward technique, requiring only ULA-plates and PHH suspension to form homogenous spheroids. Fascinatingly, 3D PHH spheroids remain viable and fully

functional in culture for at least five weeks.[12] Omics analyses revealed that 3D PHH retains transcriptomic and proteomic fingerprints similar to freshly isolated hepatocytes.[12,91] Moreover, 3D PHH spheroids are fully metabolically functioning and provide highly reproducible results in long-term ADMET studies.[15] Additionally, 3D PHH spheroids could be scaled to multi-well array culture, composed of 80 or more smaller spheroids cultured in one well of a plate, enabling accurate quantification of turnover of the compounds with low-clearance values.[92] Lastly, 3D PHH spheroids are compatible with the high throughput screening (HTS) format, which is particularly useful in ADMET screens of leading drug candidates.[93] However, not all PHH donors form 3D spheroids, and it has not yet been demonstrated that pooled PHH batches from multiple donors (commonly sold by life science providers) could form spheroids.

Lastly, hepatic microphysiological devices, also known as liver-on-a-chip platforms (Figure 5f), are used to mimic *in vivo* physiological conditions in *in vitro* setting.[62,94] For instance, perfused systems facilitate the formation of oxygen and nutrient gradient and the formation of a zonal pattern similar to the one observed in the liver lobules.[95,96] Moreover, microphysiological devices are used to recapitulate multiple organ interplay *in vitro*. For example, on-chip co-culture experiments of 3D PHH spheroids and intact primary human islets demonstrated that insulin secretion and hepatic insulin response could be emulated successfully *in vitro*.[97] However, high costs, robustness issues in device production, as well as complicated handling and necessity to purchase extra equipment for chip platforms maintenance, are currently limiting wide integration of liver-on-a-chip platforms in drug disposition studies.[94]

To sum up, there are a number of ways to culture PHH and individual investigators should select a culture configuration fitting their research aim and resources. The focus of this doctoral thesis is on the characterisation and development of 3D PHH spheroids. This culture approach was chosen on the grounds of high physiological relevance, possibility of maintaining culture long-term, high-throughput format compatibility, and 3Rs relevance.

3D PHH culture workflows

To guide the reader through the work presented in this doctoral thesis, it is useful to briefly describe a typical workflow associated with culturing 3D PHH (Figure 6). To obtain spheroids, PHH from selected donors are seeded in ULA plates (Table 4) the culture medium (Tables 2 and 3) complemented with 10% foetal bovine serum (FBS). Left undisturbed, PHH self-assemble into 3D PHH. The process takes between 3 to 10 days depending on the donor. Once and if — not all PHH donor batches will form spheroids — 3D PHH are formed, they could be used for further experiments and molecular analysis, for instance: assessment of DMEs and transporters activities,[98,99] prediction of drug-induced hepatotoxicity[14] or identification of novel therapeutic

targets.[100] Of note, at this point, functional and phenotypical evaluation of 3D PHH should be performed to ensure the correct interpretation of experimental results obtained using this *in vitro* model.

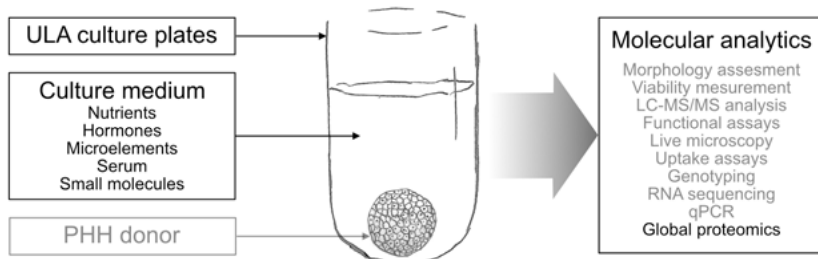

Figure 6. Constituents of 3D PHH cultures. Highlighted components are discussed in this thesis.

Phenotypic description using mass-spectrometry based global proteomics

Truthful characterisation of cellular and molecular processes is one of the ultimate goals of life sciences. For that, a thorough understanding of phenotypes is essential. With the technological advances in biomedical fields, it has become increasingly clear that functional diversity of the cells, organs and organisms stems from the proteome level.[101] Whilst genome of a species typically remain stable, transcriptome is susceptible to dynamic change in response to internal or external stimuli. This variance in protein-coding mRNAs can reasonably explain protein expression changes at the global level.[102–104] However, transcript levels by themselves are not sufficient to predict protein abundances.[105,106] In addition to that, proteins are the main targets of most drugs and ADMET-related proteins directly modulate drug fate in the body.[107,108] Therefore, analytical methods to characterise cellular proteomes are of great value for drug discovery and development.

Mass spectrometry (MS)-based proteomics analysis is a powerful analytical method for studying protein expression on a global scale. Recent technological advancements in the proteomics field enables uncomprehensive spatial and temporal resolution of proteomes.[109–111] MS technology provides an unmatched versatility in approaches to study proteins. In this doctoral thesis, the most common quantitative untargeted label-free bottom-up proteomic experiments using data-dependent acquisition (DDA) is used. This workflow is excellently described elsewhere.[112] In a nutshell, proteins are extracted from the cells and digested into peptides, that are analysed using LC-MS/MS (Figure 7). Subsequently, raw MS data is processed using software packages,[113–115] and the obtained protein expression values are used for downstream statistical

analysis and biological interpretation of the obtained results. Of note, the ingenuity of this approach is well summarized in the phrase “bottom-up”, that refers to the subsequent inference of information about the proteins from peptide analysis, which is analytically easier than the analysis of untouched proteins.

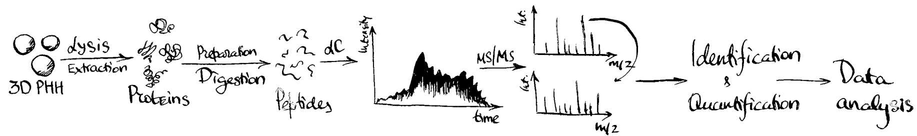

Figure 7. A typical bottom-up proteomics workflow. First 3D PHH are lysed and sonicated for protein extraction. Proteins are then reduced, alkylated and enzymatically digested into peptides that are separated by liquid chromatography (LC), and analysed with tandem mass spectrometry (MS/MS). The data is processed for peptide and protein identification and quantification, and these results are later on used for bioinformatics analysis.

Aims of the thesis

The overall aim of this thesis was to perform standardisation and functional benchmarking of 3D primary human hepatocyte spheroid cultures as a reliable *in vitro* model for drug development.

The specific aims were:

- To characterize the variability observed in 3D PHH culture conditions, and optimize 3D PHH cell culture medium to mimic physiologically relevant environment (Paper I).

- To investigate whether the developed physiologically relevant 3D PHH cultures could be successfully applied for drug disposition studies (Paper II).

- To evaluate the performance of different ULA plates in regards to spheroid formation and functional performance of 3D PHH long-term cultures (Paper III).

- To compare the performance of mass spectrometry-based global proteomic workflows and their applicability for phenotypic description of 3D PHH *in vitro* cultures (Paper IV).

- To transition 3D PHH to animal products-free chemically defined conditions, and benchmark the utility of these cultures for preclinical drug development (Paper V).

Methods

Human liver tissues and hepatocyte isolation

The liver tissue for hepatocyte isolation was obtained from cancer patients undergoing liver resection surgery at Uppsala University Hospital (Akademiska sjukhuset). All donors signed an informed consent form in agreement with the approval from the Uppsala Regional Ethical Review Board (Ethical approval no. 2009/028 and amendment 2019/1108). The acquired liver tissue surplus (essentially surgical waste) pieces were histologically normal.

Primary human hepatocytes (PHH) were used throughout all projects. Cells were isolated in-house from the patient liver tissue, with the exception for Paper IV, when PHH were purchased from BioIVT (Brussels, Belgium). Human hepatocytes were isolated using a modified version of the previously described perfusion and digestion procedure.[116] In brief, smaller liver pieces were immediately snap-frozen for biobanking; while the biggest liver tissue piece was flushed with Hypothermosol FRS (Biolife Solutions) directly in the surgical room and quickly transported on ice to the laboratory. Right after arriving at the laboratory, the two largest blood vessels were cannulated, while the unencapsulated edge of the liver piece and the remaining blood vessels were sealed using surgical glue. Afterwards, liver tissue was submerged in warm PBS and first perfused with EGTA buffer to remove Ca^{2+} ions and disrupt the tight junctions between the cells. Next, the liver piece was perfused with a digestion buffer containing collagenase, which breaks down the extracellular matrix and releases hepatocytes from surrounding tissue. After around 15-20 minutes of digestion, the liver piece was transferred into a sterile container. The Glisson capsule was opened to release liver cells suspension, which was subsequently cleaned up in funnel filters and Percoll (GE Healthcare, Chicago, Illinois) centrifugations. Isolated PHH were cryopreserved in CryoStore CS10 solution supplemented with 10% FBS using isopropanol-assisted controlled freezing at -80° C and stored at -150° C until use.

3D PHH cultures

In brief, cryopreserved hepatocytes were gently thawed and transferred to isotonic 27% Percoll in FBS supplemented (or plain in Paper V) culture medium and centrifuged at 100 g for 10 minutes. After the centrifugation, supernatant

with cell debris and dead cells was discarded, and hepatocytes were resuspended in a warm culture medium (Table 2) supplemented with 10% FBS (or serum substitute in Paper V). The cells were seeded in 384-well plates or 96-well ultra-low attachment (ULA) plates (Table 4), which were then centrifuged at 100g for five or ten minutes to bring the cells together. Plates were thereafter incubated at 37°C, 5% CO2 and 100% humidity, and spheroids formed through self-assembling. Then, the medium was gradually changed to one of the chemically defined alternatives (Table 2) without any serum supplementation. The first medium change was typically performed after the initial spheroid aggregation (within 3 to 5 days after the PHH seeding), and after that the medium change was performed every 48 to 72 hours.

Culture medium

A variety of cell culture media was used to maintain primary human hepatocytes. The key characteristics of the PHH culture media are summarised in the Table 2. In a nutshell, two commercial media with undisclosed content, Hepatocyte Maintenance Medium (HMM)[91] and Cellartis Power Primary HEP Medium (PPM)[91], as well as three conventional media with well-defined contents, Williams´ E medium (WE)[91,99,117,118] and Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F12 or DF)[91] and DMEM, were used in the projects. DMEM medium was used in original high glucose composition for the suspension and formation of spheroids only (Paper I and II).[91,99] Williams´ E and DMEM/F12 media were used in three modifications: standard hyperglycemic composition – HG (11.1 and 17.5mM glucose, respectively with addition of 10,00 ng/mL insulin), normoglycemic composition – NG (5.5mM glucose and 0.58 ng/mL insulin) and normoglycemic version supplemented with zinc – NG+. The added concentrations of the other standard PHH supplements, for instance, dexamethasone (0.1 µM), PEST (100 u/mL and 100 µg/mL for penicillin and streptomycin respectively), L-Glutamine (2 mM), sodium selenite (5 ng/mL), transferrin (5.5 µg/mL) — remained stable, unless otherwise specified by the medium vendor.[91]

Of note, as indicated on Figure 6, to facilitate the spheroid formation and decrease cellular stress caused by thawing, 10 % FBS is typically added to the suspension and attachment medium. In Paper V, FBS was substituted with a serum substitute cocktail developed by Rafnsdóttir et al, containing recombinant proteins, hormones, growth factors etc. (Table 3; Paper I, Supplementary Table 5).[119] This defined animal-free mixture was used only as an alternative to FBS, otherwise PHH were cultured according to the standard workflow, namely — formation with FBS or animal-free supplement and plain culture medium afterwards.

Table 2. Cell culture media used in the projects, and their composition and concentration of the key components.

Medium	Vendor	Catalogue number	Application	Paper	Glucose, g/L (mM)	Insulin, ng/mL	Zn ($ZnCl_2$), mg/mL	FBS, %
HMM	Lonza	1167-5450	Culture medium	I	2 (11.1)	10,000	NS	NS
PPM	TaKaRaBio	Y20020	Culture medium	I	0.97 (5.4)	6,000	NS	NS
WE_{HG}	PanBioTech	P04-29050S4	Culture medium	I	2 (11.1)	10,000	NS	NS
WE_{NG}	PanBioTech	P04-29050S4	Culture medium	I, II, V	0.99 (5.5)	0.58	NS	NS
WE_{NG+}	PanBioTech	P04-29050S4	Culture medium	I	0.99 (5.5)	0.58	1	NS
DF_{HG}	PanBioTech	P04-41505	Culture medium	I	3.15 (17.5)	10,000	NS	NS
DF_{NG}	PanBioTech	P04-41505	Culture medium	I	0.99 (5.5)	0.58	NS	NS
DF_{NG+}	PanBioTech	P04-41505	Culture medium	I	0.99 (5.5)	0.58	1	NS
William's E	Gibco	A1217601	Culture medium	III, IV	2 (11.1)	10,000	NS	NS
DMEM	Invitrogen	21063	SA	I, II	4.5 (25)	10,000	NS	10
WE	PanBioTech	P04-29050S4	SA	II, V	0.99 (5.5)	0.58	NS	10

NS = not supplemented; SA = suspension and attachment.

Table 3. The serum substitute cocktail composition.[119]

Compound		Concentration in medium, mg/L
Inorganic salts	Na_2SeO_3/H_2SeO_3	0.008
Fatty acids	Linoleic acid	1
	Lipoic acid	0.05
Vitamins	L-Ascorbic acid	0.012
	Calciferol	0.025
	Choline chloride	3.5
	Folic acid	0.33
	myo-Inositol	4.5
	Thiamine hyrdrochloride	0.08
	α-tocopherol phosphate	0.003
	4-Aminobenzoic acid	0.012
	Vitamin B12	0.35
Hormones	Triiodothyronine	0.0000002
	17-β Estradiol	0.0000005
	Hydrocortisone	0.00025
Proteins and Growth Factors	Transferrin	50
	bFGF	0.001
	Collagen	0.1
	EGF	0.01
	Fetuin	0.04
	iGF1	0.005
	Laminin	0.02
	pDGF	0.002
	Vitronectin	0.1
	Human serum albumin	1250
Other	Glutathione	0.012
	All-trans retinoic acid	0.025
	Cholesterol	0.05
	Hypoxantine sodium	1.75
	O-Phosphoryl ethanolamine	5
	Pyruvate	110
	Ribose	0.125
	Xantine	0.085
	Uracil	0.075

Concentrations are provided as mg/L in suspension and attachment medium.

ULA plates

Ultra-low Attachment (ULA) plates are indispensable 3D cell culture tools, that promotes the self-assembly of scaffold-free spheroid microcultures. Throughout all projects ULA plates were used to form 3D PHH and an overview of the suppliers and architecture of the used ULA plates is provided in Table 4.

Table 4. Overview of the low attachment plates used in the papers.

Plate	Vendor	Cat. number	Paper	Number of wells
Costar Multiwell ULA	Corning	CLS7007	II-V	96
Corning Spheroid Microplate	Corning	CLS4516	I, II	384
BIOFLOAT 96	faCellitate	F202003	III	96
BIOFLOAT 384	faCellitate	F224384	III	384
CELLSTAR 96	Greiner BIO-ONE	650970	III	96
CELLSTAR 384	Greiner BIO-ONE	787979	III	384
CellCarrier ULA	PerkinElmer	6055330	III	96
Phenoplate ULA	PerkinElmer	6057800	III	384
Nunclon Sphera	ThermoFisher	174925	III	96
PrimeSurface 3D	S-Bio	MS9384UZ	III	384

Viability

PHH viability was assessed via ATP measurement using the CellTiter-Glo 3D assay (Promega, Madison, Wisconsin) according to the manufacturer's instructions. Briefly, the PHH plates and CellTiter-Glo® 3D reagent were left for 30 minutes at room temperature for equilibration. Subsequently, CellTiter-Glo® 3D reagent was added to the well in a volume equal to the cell culture medium, and the plate was incubated at the orbital shaker for 30 minutes more. The luminescence was measured on a plate reader using 1 s integration time for at minimum number of four technical replicates/wells.

Albumin secretion

Albumin secretion (expressed as pg/cell/day) was used to measure liver function of the PHH spheroids. Human albumin concentration was quantified using a human albumin ELISA kit (Thermo Fisher Scientific) according to the manufacturer's instructions. The *in vivo* production span of human albumin was calculated to be 43 pg/day/cell during dormancy production and 230 pg/day/cell during maximal production; using 9 g/day as dormancy level and 48 g/day as maximum production level,[120] 139 million cells/g liver,[121] and a liver weight of 1.5 kg.[19]

Immunohistochemistry

Immunohistochemistry was performed to assess the morphology of spheroids and spatially expressed markers of hypoxia, apoptosis and lipid accumulation. PHH spheroids were prepared using a standard immunohistochemistry protocol. Briefly, for hypoxia staining, spheroids were first incubated with 200 mM pimonidazole for one hour before fixation. For the other stainings, PHH spheroids were washed three times in PBS and fixed in 4% formaldehyde in PBS overnight, then embedded in paraffin and sectioned at a thickness of three micrometers. The staining was performed using DAKO staining kits and is described in details elsewhere.[91] Primary anti-caspase-3 (apoptosis), anti-adipophilin/Plin2 (lipid droplets formation) and anti-Ki-67 (proliferation) antibodies were used along with pimonidazole (hypoxia) staining.[122–125] The slides were then scanned using the automated scanning system Nanozoomer S60 (Hamamatsu; Sunayama-cho, Japan) and saved as high-resolution NDPI images. The images were converted to .tiff files using the open-source cross-platform software NDPITools and analyzed using ImageJ imaging analysis tool.[126,127] Quotients of positively stained areas (coloured in brown) in pixels were calculated in at least five spheroids using a colour threshold adjustment method, and normalized with spheroid areas also measured in pixels. Colour threshold was kept consistent between all samples.

Hepatic CYP metabolism

Compound exposure

The metabolite formation rate was measured to assess the metabolic capacity of the PHH spheroids. Spheroids were incubated for 4 to 8h (depending on the study) with a cocktail of prototypical CYP substrates (Table 5). The reactions were stopped with an ice-cold acetonitrile solution containing internal standard (warfarin); and cell culture medium with metabolites (with or without spheroids) was immediately frozen and stored at -80°C until LC-MS/MS analysis.

Table 5. Prototypical CYP substrates and their concentrations used in the studies.

CYP	Substrate	Concentration µM	Metabolite	Paper
CYP3A4	midazolam	5 or 10	1-hydroxymidazolam	I and III, V
CYP2D6	dextromethorphan	5	dextrorphan	III
	bufuralol	5	1-hydroxybufuralol	I, V
CYP2C9	diclofenac	5 or 10	4-hydroxydiclofenac	I and III, V
CYP2C19	omeprazole	10	5-hydroxyomeprazole	I,
CYP2C8	amodiaquine	10	N-desethylamodiaquine	III
CYP2B6	bupropion	10	hydroxybupropion	V

LC-MS/MS quantification

The samples were diluted with ice-cold acetonitrile/water and primary metabolites (Table 5) were measured using an Acquity UPLC (Waters Corp, Milford, Massachusetts) with a C18 column coupled to either SCIEX Q-TRAP 6500 (Framingham, Massachusetts) or Waters TQ-S Micro (Milford, MA). Absolute quantification of substrates and metabolites using normalisation to internal standard and a calibration curve using MultiQuant or TargetLynx software packages for SCIEX and Waters instruments respectively. Specific details of chromatographic events and MS transitions can be found in the relevant papers (Table 5).

Transporter kinetic studies

To showcase the possibility of performing transporter kinetics in 3D PHH spheroids, uptake of the 4-(4-(dimethylamino)styryl)-N-methylpyridinium (ASP+) fluorophore by Organic Cation Transporter 1 (OCT1) was evaluated. PHH spheroids were used after full spheroid formation (typically one week of culture). Prior to the fluorophore uptake assay, PHH culture medium was exchanged to warm Hank's balanced salt solution (HBSS). The PHH plate was centrifuged at 100 g for 30 s and incubated at 37° C in the HBSS for 10 minutes. Next, HBSS containing ASP+ was added, giving a final ASP+ concentration of 1-50 µM, the plate was centrifuged at 100 g for 30 s, and fluorescence from ASP+ was measured in each spheroid at regular intervals for up to 60 min. The wavelengths were set to λ_{ex} = 475 nm and λ_{em} = 605 nm in a Tecan Spark or a CLARIOstar microplate reader. The gain adjustment setting was kept off independently of the machine to ensure the compatibility of the data when several culture plates were used in the assay.

OCT1 kinetics calculations were performed as previously described for SLC transporters.[128,129] The uptake of ASP+ in PHH spheroids was linear for up to 20 minutes in the concentration range of 0.5-50 µM. To assess the uptake kinetics of ASP+, the initial uptake rate derived by the linear regression equation was plotted against the ASP+ concentration (1-50 µM). K_m and V_{max} values were determined by non-linear regression according to the Michaelis-Menten equation (1) using Prism version 9. (GraphPad, San Diego, CA), where V is the uptake rate, V_{max} is the maximal uptake rate (at saturating substrate concentration), [S] is the substrate concentration, K_m is the substrate concentration at which the uptake rate is half of V_{max} (Equation 1).

$$(1)\ v = \frac{v_{max}\, S}{K_m + S}$$

Transporter inhibition

Transporter inhibition studies were performed in the 3D PHH spheroids for the OCT1 transporter. The inhibition assay was performed based on the

previously published method.[129] Prior to inhibition experiments, the culture medium was changed to HBSS containing none or one of the OCT1 inhibitors, namely ketoconazole, verapamil, clomipramine, diltiazem, clotrimazole or chlorpromazine to give a final concentration of 100 µM. The plate was centrifuged at 100 g for 30 s and incubated at 37°C for 10 minutes. Next, HBSS with the corresponding inhibitor and ASP+ was added to a final ASP+ concentration of 1 µM and the plate was centrifuged at 100 g for 30 s. The fluorescence was measured as described in the section above. Fluorescence read out from the PHH spheroids incubated only with ASP+ was used to calculate the inhibitory potential of the compound tested. The inhibition was calculated as a percentage of the maximal ASP+ uptake rate.

Long-term xenobiotics exposure

The PHH spheroids were incubated with the compounds for one or two weeks, and the culture medium was changed every 48 to 72 h to ensure the replenishment of the nutrients and repeated exposure to xenobiotics. Compound stock solutions were prepared in dimethyl sulfoxide (DMSO) and subsequently diluted in the serum-free cell-culture medium to a final DMSO concentration of at maximum 0.1%. The paracetamol stock was dissolved in HBSS or in cell medium. The compound exposure concentrations were determined using the approach developed by Vorrink et al.[14] In brief, therapeutic exposure concentrations (C_{max}) were obtained from literature, and PHH spheroids were exposed to one, five or twenty times C_{max} concentration of selected compounds. The viability of the 3D PHH was evaluated using the described CellTiter-Glo 3D assay. For proteomics analysis spheroids were collected and snap-frozen in liquid nitrogen for further use.

Global proteomics analysis

Sample preparation

Filter Aided Sample Preparation (FASP) — Paper I

Samples were lysed and sonicated and the final concentration of the lysates contained 50 mM ditiotreitol (DTT) and 2% sodium dodecyl sulfate (SDS) in 100 mM Tris/HCL at pH 7.8. Cell lysates were digested using sequential proteolytic digestion with trypsin and LysC according to the Multiple enzymes for sample digestion Filter Aided Sample Preparation (MED-FASP) protocol.[130] Protein and peptide concentrations were quantified using the tryptophan fluorescence method.[131]

Solid-phase-enhanced sample-preparation (SP3) — Paper II

Samples were lysed in 25 mM HEPES buffer, pH 7.6, containing 4% SDS and 1 mM DTT, and proteins were denatured at 95°C for 5 minutes. The sample preparation procedure was performed using a modified single-pot, solid-phase-enhanced sample-preparation (SP3) technology.[132] In brief, samples were sonicated, and proteins were alkylated, bound to the beads and digested overnight with trypsin and LysC digestion enzymes. The following day the peptide digests were cleaned up on the beads and subsequently cleared from the beads by using a magnetic force, transferred to the glass LC-MS and dried in the Speedvac at 40°C. Protein and peptide concentrations were determined by fluorometric quantification with the Protein Broad Range Assay on the Qubit 4 Fluorometer (Thermo Fisher Scientific, Waltham, MA).

ProteaseMAX/urea (PMU) or RIPA lysis — Paper IV

In paper IV various sample preparation protocols were compared. For lysis in PMU, spheroids were suspended in 1M urea and 0.1% ProteaseMAX (Promega) in 50 mM Tris-HCl, pH 8.5 and 10% acetonitrile as well as protease and phosphatase inhibitors (Pierce). The samples were sonicated in a water bath for 10 min with a VibraCell probe (Sonics & Materials) at 10% amplitude. Lysates were spun down at 13,000 g at 4°C for 10 min.

For lysis in RIPA buffer, spheroids were centrifuged at 4°C in a RIPA buffer at 13,000 g for 10 min before precipitation with acetone overnight at −20°C. Acetone was evaporated at room temperature (RT) for 30 min and the protein pellets were dissolved in a 8M urea buffer, sonicated and diluted in 50 mM Tris-HCl buffer. Afterwards, for both sample preparation methods, proteins were reduced with DTT, alkylated with iodoacetamide and digested with trypsin overnight. The digestion was stopped with formic acid, samples were cleaned on a C18 HyperSep plate (Thermo Fisher) and dried under vacuum using a Vacufuge concentrator (Eppendorf). Samples for tandem mass-tag (TMT) labelling were reconstituted in 50 mM triethylammonium bicarbonate (TEAB), supplemented with TMT-10plex (6 tags only) reagents and incubated at room temperature for 2 h (Thermo Fisher). The labelling reaction was stopped by adding hydroxylamine and six individual samples (TMT channels) were combined to one analytical sample and dried in vacuum, followed by clean up on a C18 HyperSep plate.

Thermal lysis — Paper IV

For thermal lysis, samples diluted with 100 mM TEAB, pH 8 were subjected to four cycles of freezing in liquid nitrogen for 2 min and thawing at 70°C on a heating block for 2 min. Proteins were then denatured at 90°C for 5 min and cooled to RT before further processing. Then proteins were directly digested by adding trypsin and incubating over night at 37°C. For TMT labelling, samples were then supplemented with TMT-10plex reagents and incubated at

room temperature for 2 h. Labelling was stopped by adding 1.7 µL of 5% hydroxylamine and incubation for 15 min at RT. TMT-labelled peptides were combined directly into a sample vial and dried in vacuum.

PreOmics kit — Paper V

Samples were rapidly defrosted and proteins were prepared using the iST sample preparation kit (PreOmics, München, Germany) according to the instruction manual with the exception of the reconstitution buffer that was exchanged to 0.1% formic acid in LC-MS/MS grade water. Then samples were transferred to glass LC-MS vials and dried in a Speedvac at 40 °C. Final peptide concentrations were determined by fluorometric quantification with the Protein Broad Range Assay on the Qubit 4 Fluorometer (Thermo Fisher Scientific, Waltham, MA).

LC-MS/MS analysis

In all papers, peptides were reconstituted in a 0.1% formic acid solution in LC-MS/MS grade water and separated on an EASY-spray C18-column (50 cm, 75 µm inner diameter), using an acetonitrile/water gradient (0.1% formic acid) at 300 nL/min. Eluted peptides were analysed using the TopN method (full MS followed by ddMS2 scans) on an Orbitrap Q Exactive HF mass spectrometer (Thermo Fisher Scientific), operating in a data-dependent mode. MS tune parameters are described in details in the respective papers.[91,99,118]

For all projects, raw data files are uploaded to the ProteomeXchange Consortium via the proteomics identification database (PRIDE).[133] In compliance with Findable, Accessible, Interoperable, and Reusable (FAIR) data principles, for Papers II and V raw files were supplemented with Sample and Data Relationship Format (SDRF) annotation file.[134–136]

Data analysis

In papers I, II, and V, raw MS datafiles were processed using MaxQuant software.[113,137,138] Proteins were identified by searching MS data of peptides against a reference human proteome database retrieved from the UniProtKB.[139]A detailed description of the parameters used for peptide identification and integration by MaxQuant can be found in the mqpar.xml files uploaded to the PRIDE repository (separately for each project). Briefly, carbamidomethylation was set as fixed modification and oxidation and acetylation as variable modifications; match between runs (MBR) and label-free quantification (MaxLFQ) algorithms were used. The decoy sequences were created by reversing the target sequences. Peptide-spectral matches, peptides and proteins were validated at a 1% FDR estimated using the decoy hit distribution. Quality control of the MaxQuant search was performed using an R-based QC pipeline called Proteomics Quality Control (PTXQC).[140] Subsequent data

clean-up was performed using an in-house developed proteomics data pipeline in R or Perseus software package.[141] Then protein abundances (fmol/µg total protein) were calculated with the Total Protein Approach (TPA).[142] Proteins related to the drug disposition were extracted by subsetting against the ADMET-related genes list.[143]

In paper V, raw data files were analysed using Proteome Discoverer v2.5 (Thermo Fisher) with the MS Amanda 2.0 search engine against the human protein database (SwissProt) retrieved from the UniProtKB. Briefly, a maximum of two missed cleavage sites were allowed for full tryptic digestion, carbamidomethylation of cysteine was specified as a fixed modification except for freeze-thaw samples, while TMT6plex on lysine and N-termini (+229.1629 Da) for TMT labelled samples, oxidation on methionine as well as deamidation of asparagine and glutamine were set as dynamic modifications. Initial search results were filtered with 5% FDR using the Percolator node in Proteome Discoverer.

In all cases, subsequent data analysis was performed using a plethora of tools that are stated in every paper separately. For instance, gene ontology (GO) analysis was performed using Panther or Gorilla GO tools.[144,145]

Results and discussion

The choice of medium affects 3D PHH morphology and ADMET properties (Paper I and II)

As described in details in the introduction section, 3D PHH are emerging as a gold-standard *in vitro* model for culturing primary human hepatocytes. The establishment of these cultures is fairly straightforward. However, a plethora of different culture conditions are used for 3D PHH. For instance, cell culture media for 3D PHH ranges from well-defined traditional media like Williams´E (WE),[12,146] Dulbecco's Modified Eagle Medium/Nutrient Mixture F12 (DMEM/F12, further referred as DF),[147] to numerous commercial alternatives with undisclosed contents and high cost.[78,148] These media vary in the levels of nutrients and supplements (Table 2), and the exact effect of these on 3D PHH cultures has not been investigated.

In paper I, the influence of the cell culture medium on the long-term 3D PHH spheroid cultures was studied. After establishing spheroid cultures in a 384-well format; morphology, functional performance and proteomes of 3D PHH cultured in eight different media (Table 2; Paper I, Table 1) were thoroughly evaluated. Of note, conventional cell culture media are often hyperglycemic, with a glucose concentration of 11 mM and above and an insulin concentration of 10,000 ng/mL, whilst physiologically normal fasting state levels range between 3.9 to 5.6 mM glucose and 0.19–1 ng/mL for insulin.[149–151] Therefore, we modified the content of well-defined traditional media by reducing the glucose and insulin to fasting levels, and named these media normoglycemic (NG). Lastly, we hypothesized that by reducing insulin addition — typically comes in insulin-zinc solutions — we deprive PHH of essential zinc supplementation.[152,153] Therefore, we complemented normoglycemic media with additional zinc (NG+ media).

3D PHH cultured in different media vary noticeably in performance

First, we observed that PHH from four biological donors formed satisfactory 3D PHH is all eight cell culture media. Then, immunohistochemistry was used (Figure 8a) to allow a more detailed morphological assessment of 3D PHH. In contrast to the light microscopy, spheroids that were cultured in DMEM/F12

media showed asymmetric and fragile 3D structures compared to those in other media. Interestingly, in subsequent proteomics analysis we observed that in DF-cultured spheroids, proteins associated with the actin cytoskeleton were downregulated (Figure 8e). Amongst these are Arp2/3 complex, a protein that binds and initiates growth of new actin filaments, and Plastin-3, that facilitates formation of F-actin bundles. Interestingly, analysis of the medium composition provided a possible explanation to these observations — the DF media lack ascorbic acid. Ascorbic acid is involved in pathways important for a functioning cytoskeleton and cell junctions, e.g., collagen synthesis, actin regulation, and microtubule stabilization, thus the lack of this supplement could have reflected poorly on the spheroid morphology.[154,155]

Further, we measured albumin secretion and ATP content, as a proxy for cellular health state and viability.[12,14] The average albumin secretion (for four donors over three weeks in eight media) was within the normal range observed *in vivo* (Figure 8b), and slightly higher in WE cultured 3D PHH. No systematic differences in albumin secretion were observed between the hyper- and normoglycemic media. Further, we observed that two commercial media (HMM and PPM) had spheroids with the highest ATP content, followed by the WE media (Figure 8c). Interestingly, the glucose supplementation (Table 2) in the media was not determinant of the ATP content.

Next, we investigated the effect of the different media on the global protein expression. Freshly thawed PHH from four donors were used as a reference in this analysis, assuming that these cells exhibit phenotypes closest to the native *in vivo* hepatocytes. Principal component analysis showed that the proteomes distributed into two distinct clusters along the first principal component. Interestingly, PHH spheroids cultured in PPM medium formed one separate cluster, whereas all other media and reference proteomes formed the other cluster (Figure 8c). Global proteomics analysis also provided a beautiful insight into the 3D PHH formation process. During the first week of culture we observed consistent upregulation of proteins involved in ECM formation and cell-cell, as well as cell-ECM interactions (Figure 8e). Notably, this phenomenon was observed elsewhere[73] for 3D PHH cultures and repeated in all following 3D PHH proteomics analyses in our hands, e.g. Paper II and V.

As described in the introduction, PHH cultures are an important tool in studies of drug metabolism and toxicity. Thus, an in-depth analysis of drug-metabolizing and other ADME-related proteins in the 3D PHH is essential. Of 682 ADME-related proteins,[143] 315 were found in the proteomes of the analysed PHH spheroids and sorted according to function using the PANTHER classification system.[144] As expected, the two most prevalent protein classes were metabolite interconversion enzymes and transporters (26 and 16% of identified ADME-related proteins respectively). Of note, most of the ADMET proteins were stably expressed over time in spheroids from all media except for PPM (Figure 8f; Paper I, Figure 5), whilst differences in glucose and insulin levels between media had no impact on ADME-related proteins.

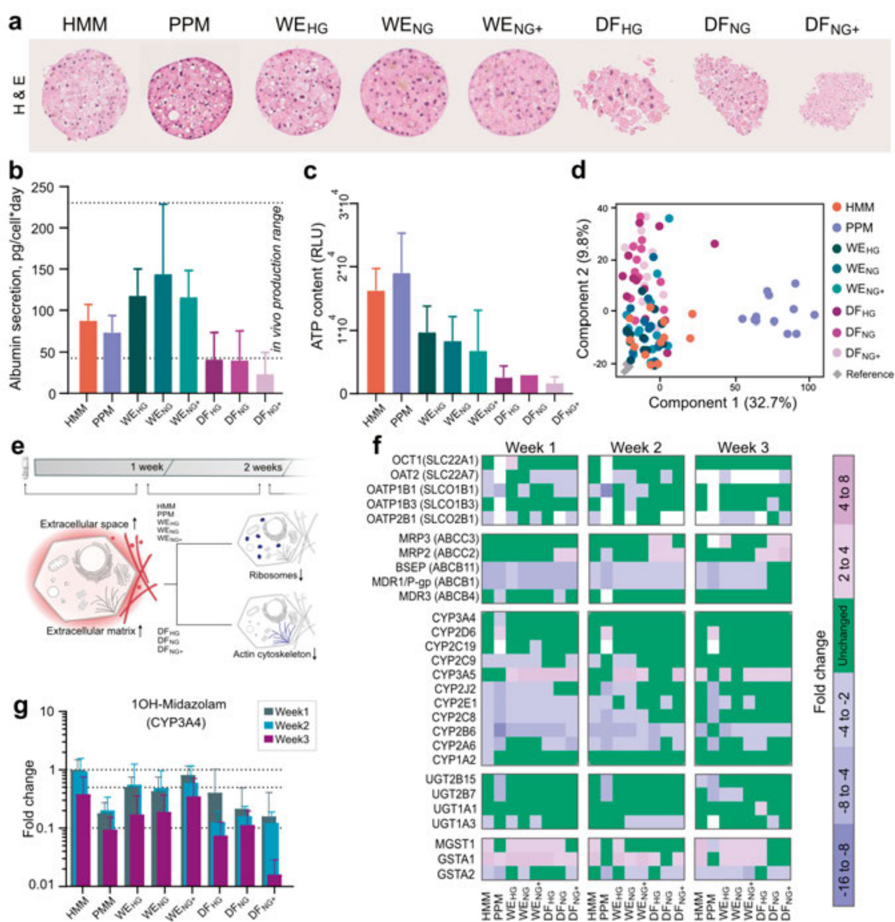

Figure 8. The formation, protein expression, and function of 3D PHH from four donors were followed for three weeks in 384 well format in eight culture media (composition of media provided in Table 2). **a.** Morphology of 3D PHH spheroids cultured in eight different media. Haematoxylin and eosin staining (H & E). **b.** Average albumin secretion over three weeks of culture. Albumin production *in vivo* ranging from dormant to maximal albumin levels (marked range) was calculated from literature data. See methods section for details. **c.** Average ATP content over three weeks of culture, provided in absolute relative fluorescence units. **d.** Principal component analysis of the global proteomes of 3D PHH spheroids cultured in different media at one, two and three weeks of culture from four donors. The number in parentheses is the percentage of variability explained by each component. Reference = uncultured and freshly thawed cryopreserved PHH. **e.** Statistical enrichment analysis for cellular components: a selection of up- and downregulated cellular components (in red and blue colours respectively). Each arrow represents the change between two time points: cryopreserved and cultured for 1 week; cultured between 1 and 2 weeks. Only cellular components with a false discovery rate below 0.05 were considered significant. See Figure S1 in Paper I for a comprehensive overview of all significant cellular components. **f.** Expression of clinically relevant drug transporting and drug metabolizing proteins. Expression values are normalized to the reference. **g.** Hydroxy midazolam formation was measured using LC-MS/MS and compared to the individual reference.

Next, clinically relevant transporters and DMEs were investigated. Briefly, variable expression pattern was observed for the clinically relevant transporters (Figure 8f), and 3D PHH cultured in PPM did not express detectable levels for most of the clinically relevant SLC transporters. Notably, OCT1/SLC22A1, which transports important cationic drugs such as metformin into the hepatocytes,[129] remained remarkably stably expressed over time in most media, while MDR1/P-gp/*ABCB1* and BSEP/*ABCB11*, were downregulated in most media. A similar transporter expression pattern was observed in 3D PHH by other researchers.[90] In terms of metabolizing enzymes, CYP expression varied and was often lower than in the reference uncultured PHH. Importantly, CYP3A4, CYPD6 and CYP2C19 were stably expressed at levels comparable to the reference in all media expect for commercial PPM. Interestingly, while there seemed to be no effect of PPM on CYP3A4, it seems to induce CYP2D6, which later on corresponded to increased hydroxy bufuralol formation in that medium. Furthermore, UGTs were stably expressed over time at levels of the reference in most cases, including PPM, and MGST1 and GSTA1 seemed to be induced in 3D PHH cultures.

Lastly, the activity of CYP enzymes that play a major role in drug metabolism, namely CYP3A4, CYP2C9, CYP2D6, and CYP2C19, was investigated using FDA-recommended marker reactions with probe substrates (Figure 8g; Figure 6 in Paper I). Generally, a time-dependent reduction in CYPs activity was observed in all media, and this phenomenon could not be explained by protein expression values of these proteins or their cofactors. In terms of cell culture media, 3D PHH cultured in DMEM/F12 media demonstrated overall lower metabolic capacity, while spheroids cultured in HMM and Willliams´ E media exhibited somewhat comparable results. The effect of PPM varied to both directions. Generally, it is exceptionally hard to deduce the activity of a single CYP from marker reactions. Even if a probe substrate is primarily metabolized by a single enzyme, drug metabolism often involves several parallel and/or downstream metabolic pathways. Further, substrate uptake and export determine the accessibility of probes to both CYP and for the measurement by researchers.[21] Lastly, the levels of unbound drug concentrations might vary significantly between *in vitro* microenvironments and cell culture media.[156–158] For instance, the apparent decrease in CYP3A4 and CYP2C9 metabolites formation in PPM can be explained by serum protein binding — PPM was discovered to be the only media to be supplemented with bovine albumin.[159]

In conclusion, we observed consistently better performance from 3D PHH cultured in HMM and WE media, whilst the use of DF and PPM medium typically decreased the performance of hepatic spheroids. Importantly, DF medium composition is disclosed, thus we were able to provide possible explanations to the observed effects. Conversely, nothing was known to us about PPM composition — a significant hindrance to the reproducibility and interpretability of the research results. *More on that topic in Paper V.*

Physiologically relevant medium is a fine alternative to the hyperglycemic counterparts

Results presented by other researchers and in Paper I clearly demonstrate that Williams´ E medium provides desired phenotype and functional performance of 3D PHH cultures.[12,15,73,90,91,160] But do 3D PHH benefit from the adjustment of glucose and insulin to physiological fasting levels? As it is apparent from Paper I and the results highlighted in Figure 8, the switch to the normoglycemic WE_{NG} did not have any significant effect on the albumin production, viability, morphology, proteomes, or expression of the proteins relevant for drug development or CYP function. Nevertheless, 3D PHH cultured in high glucose and insulin levels, developed lipid droplets and increased glycogen deposits over time. These features are not necessarily desired in 3D PHH, unless achieving a disease specific phenotype was an aim of the study.[100,161] In addition, 3D PHH cultures in physiological levels of glucose and insulin provide the possibility of studying cellular response to change in nutrients level.[97] However, the performance of 3D PHH cultured in physiological WE_{NG} as an *in vitro* model for drug development requires further evaluation.

In Paper II we assessed the applicability of 3D PHH cultured in WE_{NG} for transporter kinetics and drug-drug interaction studies. We sourced inspiration from the results obtained in Paper I (Figure 8f), and selected the stably expressed OCT1/SLC22A1 transporter for further evaluations in the following study. OCT1 transports a variety of organic cations, including endogenous bioactive amines such as choline, dopamine, and thiamine; cationic xenobiotics such as the antidiabetic drug metformin, cytostatic drugs such as oxaliplatin and imatinib, the antiviral agent lamivudine, and the opioid morphine.[162–167] Therefore, it is often recommended that drug candidates should be evaluated as substrates or inhibitors of OCT1 along with other hepatic transporters.[168]

First, the kinetics of the OCT1 transporter were studied in 3D PHH from three donors using the fluorescent model substrate and cationic fluorophore 4-(4-(dimethylamino)styryl)-N-methylpyridinium (ASP+), that has already been used in studies of OCT1 uptake and inhibition in various cell types and conventional 2D hepatocyte cultures.[129,169,170] ASP+ is a very elegant substrate for transporter studies — it exhibits binding-associated fluorescence in the membranes of living cells but not in cell culture media or common buffer systems.[171,172] This facilitated the development of an assay for quantifying the kinetics of the transporter, in which the fluorescence signal is proportional to the amount of ASP+ taken up by living hepatocytes in 3D spheroids (Figure 9a). However, the organic cation ASP+ is a shared substrate between many SLC transporters such as OCT2,3/*SLC22A2,3*; OCTN1,2 /*SLC22A4,5*; and MATE1/*SLC47A1*. None of these transporters were detected in 3D PHH used in Paper II, while OCT1 continued to exhibit a stable expression across weeks and donors (Figure 9b; Paper II, Supplementary data).

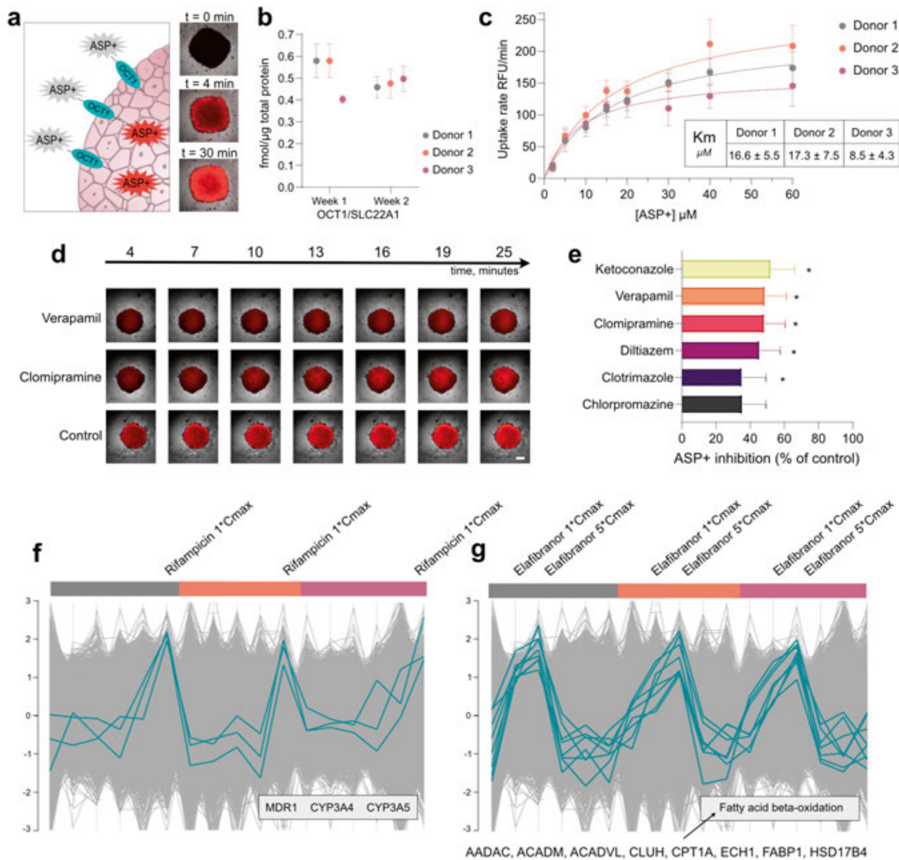

Figure 9. Spheroids cultured in physiological (normoglycemic) Williams′ E medium could successfully be used as an *in vitro* model for drug development efforts. **a.** Schematic representation of the ASP+ uptake mechanism and fluorescence microscopy visualization. **b.** Expression of OCT1 transporters in 3D PHH from three donors after one and two weeks of culture. Protein expression is in fmol/µg total protein and was measured in 3 technical replicates. The expression levels between weeks and across PHH donors were not significantly different when evaluated with differential expression analysis for global proteomics using DEqMS. **c.** Michaelis–Menten kinetics for each of the three 3D PHH donors. The points represent the mean uptake rate, and the error bars show the standard deviation of uptake rate per point, n = 8–12. **d.** ASP+ (1 µM) uptake alone and in the presence of verapamil (100 µM) and clomipramine (100 µM) in 3D PHH cultured for one week. Numbers indicate ASP+ incubation time in minutes. Scale bar = 200 µm. **e.** Uptake of ASP+ (1 µM) was measured after 5 min in the presence of 100 µM of the OCT1 inhibitors. The inhibitory effect of compounds is shown as the percentage of vehicle control, n = 8–12. All observations were statistically significant in multiple unpaired t-tests compared to vehicle control. **f and d.** Profile plots of global proteomes, highlighted proteins are upregulated in 3D PHH exposed to 1*C_{max} rifampicin and 1 and 5*C_{max} of elafibranor (on panels f and g respectively). The Y-axis represents the z-score normalized protein expression across the samples.

So, time- and concentration-dependent uptake of ASP+ was studied to determine OCT1 transport kinetics in physiologically relevant medium. Using the initial linear uptake rate, we obtained Michaelis–Menten constants (K_m) and maximum velocity value (V_{max}) for all three tested hepatocyte donors (Figure 9c), and these values were similar to those previously reported in the literature.[169,172] The accuracy of these experiments was further corroborated by the excellent agreement between kinetic experiments performed by three experimentators between 2020 and 2023 (Supplementary Table 7, Paper II). Thus, these results convincingly demonstrate that the kinetics of drug transport can be studied robustly in 3D PHH with reasonable reproducibility.

To test whether the ASP+ uptake assay could be used in drug-drug interaction studies (DDIs), inhibition of ASP+ uptake was examined using six well-established OCT1 inhibitors of various physico-chemical properties. ASP+ uptake inhibition was observed in individual spheroids using live microscopy (Figure 9d) as well as the 384-well plate reader (Figure 9e). Once again, this successfully demonstrated that 3D PHH cultured in WE_{NG} could successfully be used for *in vitro* transporter studies. However, the observed inhibition in 3D PHH was about 25% lower than previously observed in monolayers of HEK293 cells overexpressing OCT1.[172] This could be explained by slower penetration of inhibitors into the multi-layered spheroids compared to the HEK293 monolayers, and/or clearance of the inhibitors via metabolism or efflux. Nevertheless, the difference between the two rather different systems was relatively small, and the reproducible results from our screening indicate that the PHH spheroids are applicable in predictions of transporter DDIs.

Next, we investigated the sensitivity of 3D PHH in WE_{NG} to long-term drug exposure of potential OCT1 modulators. While these compounds had no or only a minor effect on OCT1 expression and function (in details described in Paper II), these pharmaceuticals caused a clear change in the expression of other proteins, either according to their target effect or know DDIs. For instance, PXR agonist rifampicin, known CYP3A4/5 and MDR1 inducer, indeed selectively induced these proteins in all three donors (Figure 9f). Moreover, elafibranor (PPARα/δ agonist) treatment led to a significant upregulation of proteins from the fatty acid beta-oxidation pathway ($1.8*10^{-3}$ q-value; Figure 9g), consistent with its mechanism of action and previously published data.[173–175] These results suggest that 3D PHH in physiologically relevant medium generate an expected response to the drug exposure and could be reliably used in drug development studies.

Again, phenotypic description by mass-spectrometry based proteomics was essential for the correct interpretation of research results. It has also provided an additional understanding of inter-donor variability in reaction to compound exposure. We observed that PHH from one of the donors expressed increased levels of LPS-binding protein, C-reactive protein, and Serum amyloid A1, suggesting an ongoing inflammatory response, possibly due to LPS contamination in this donor. *More on global proteomics for 3D PHH in Paper IV.*

The choice of plates impacts 3D PHH viability and metabolic activity (Paper III)

As was described in the introduction to this thesis and demonstrated in Papers I and II, 3D PHH formation is usually performed by aggregation in 96- or 384-well ultra-low attachment (ULA) plates. Once again, this method is rapid, scalable and does not require any additional equipment or consumables. Whilst we thoroughly evaluated the use of cell culture medium for 3D PHH, similar studies have not been conducted to compare ULA culture plates despite the wide range of available products on the market. Notably, plates from different providers vary in overall architecture, materials, well geometry and coating, and for the sake of reproducibly, should not be used interchangeably within one set of experiments.[157,176–181] To provide practical guidance for ULA plate selection for 3D PHH, we compared the performance of ten different 96- and 384-well ULA microplates.

First, PHH were seeded in ULA-plates and observed the spheroid formation efficiency in these plates. To this end, a successful 3D PHH formation was defined as PHH assembling into a single spheroid in each well without the formation of satellites or excessive cell debris (Figure 10a). Across 96-well plates, the cell suspensions aggregated predominantly into one single spheroid; while in 384-well plates, spheroid formation was more challenging and varied largely between the plate brands with Greiner384 plates resulting in formation of multiple spheroids per well (Figure 10b). Notably, this observation was consistent with our hands-on experience in years prior to this study.

Further, cell viability was assessed to ensure cellular health state and usability of 3D PHH for long-term experiments (Figure 10c). Overall, for all plate providers and architectures we observed a previously described decrease in ATP along the 3 weeks culture time.[12,15,91] However, 3D PHH cultured in Corning 96- and 384-well plates demonstrated a remarkable stability in ATP values for the whole duration of investigated period.

In the follow-up experiments some mRNA expression differences in key drug metabolizing enzymes were observed between different cell culture plates (details in Paper III). Thus, we proceeded with the evaluation of the functional activity of the CYP1A2, CYP2C8, CYP2C9, and CYP3A4 metabolic enzymes using canonical probe substrate reactions (Figure 10d-g). Oddly predictable, striking differences between CYP activities were observed between microplates. PE96 and PE384, as well as Nunc96 and Sbio384 exhibited the lowest apparent metabolic activities for all substrates, with hydroxy diclofenac (CYP2C9) and hydroxy midazolam (CYP3A4) formation being below the quantification limit. Generally, 3D PHH cultured in plates supplied from Biofloat, Corning and Greiner performed reasonably well; and Biofloat plates demonstrated the lowest variability between the different measurements. Of note, similarly to the results in Paper I, measured metabolite formation by CYP enzymes is a derivative of many parameters, including

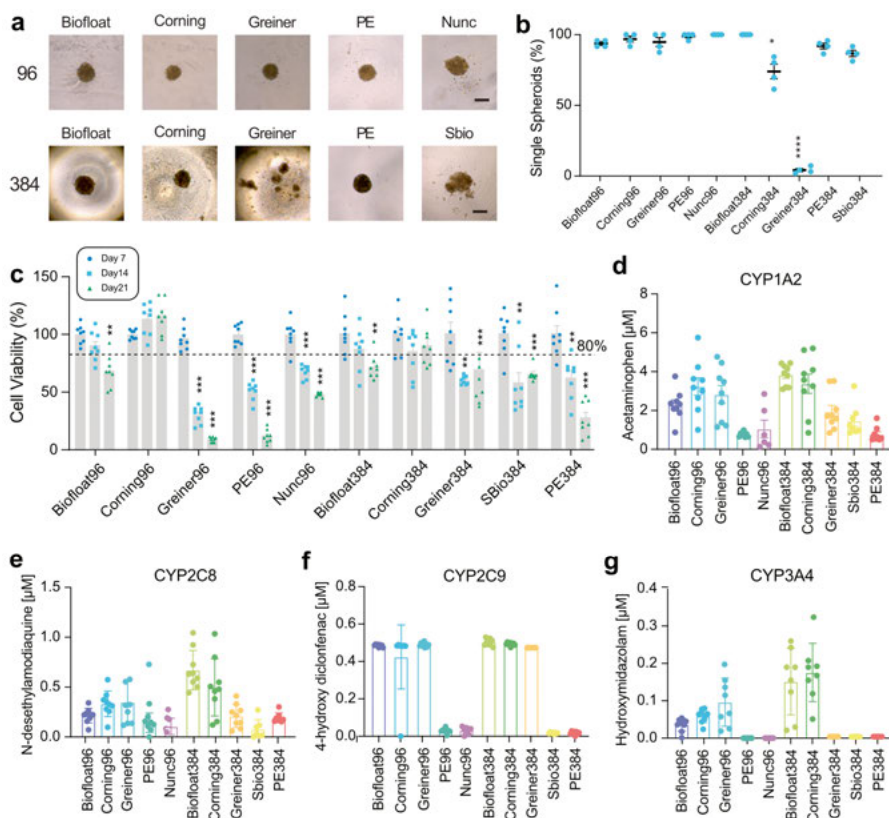

Figure 10. The selection of ULA plate supplier and microarchitecture directly affects the performance of 3D PHH *in vitro* cultures. **a.** Morphology and formation of 3D PHH in ten different ULA-plates. Brightfield images has been taken at day 7, scale bar = 200 µm. **b.** The efficiency of 3D PHH aggregation, namely a fraction of wells in which PHH aggregated into one 3D spheroid. **c.** Cell viability of 3D PHH during three weeks of culture, measured after 7, 14, and 21 days respectively, n = 8 per plate and time point. Statistical significance drawn from one-way ANOVA. The dotted lines indicate 80% cell viability that is often used to mark the range of physiologically normal variation in 3D PHH viability. **d, e, f, g.** Metabolite formation rate of acetaminophen, N-desethylamodiaquine, hydroxy diclofenac, and hydroxy midazolam, which serve measurements of the metabolic activities of CYP1A2, CYP2C8, CYP2C9, and CYP3A4.respectively.

CYP and transporter function as well as unbound drug concentrations inside and outside of the spheroids. Deducing the influence of every parameter will require an extensive set of additional experiments. Yet, the collected results altogether grant a clear possibility of informed selection of ULA-plates for 3D PHH in drug development.

To sum up, 3D PHH cultured in Corning and Biofloat supplied plates exhibited the highest spheroid formation and functional performance. *These plate brands were used in all papers summarized in this thesis.*

The choice of proteomics workflow for phenotypic description of 3D PHH (Paper IV)

As clearly demonstrated in Papers I and II, deep phenotypic description of 3D PHH using mass-spectrometry based proteomics provides a unique understanding of the utilized *in vitro* cultures and modelled conditions. Proteomics is a field of continuous and fast development, and over the years, a multitude of diverse proteomic protocols have been applied to the liver. Although different proteomics protocols have been continuously benchmarked across simulated data sets,[182] artificial standards,[183] biological fluids,[184] and human tissues,[185] studies evaluating the performance of global-proteomics protocols on *ex vivo* microtissues are lacking. In a way, the microwell format leaves spheroids being slightly too big for single cell proteomics applications, [186] and slightly too small for standard bulk analysis at sufficient resolution. These obstacles could be circumvented by using ultra-high sensitivity mass-spectrometry or culturing heaps of 3D PHH. Both of these solutions are pricey and are not routinely available to all researchers in the field. So, as one of the main users of mass-spectrometry based proteomics for phenotypic description of 3D PHH,[12,90,91,99,187] we compared the performance of different protocols for proteomic analysis of organotypic human liver spheroids, assessing the variables of cellular input (1 spheroid to whole 96-well plate), sample preparation strategies (ProteaseMAX/Urea = PMU, RIPA buffer or freeze-thaw) and quantification methods (tandem mass tags = TMT or label-free = LF). The overview of these parameters is provided in Figure 11a.

As expected, we observed that with higher initial material, the number of quantified proteins increases monotonously (Figure 11b). However, we noticed a diminishing return in that increase. For instance, a 12-fold increase in cellular input — from 8 to 96 spheroids — increased the number of quantified proteins only by 15-18%. These results suggest that cellular input can majorly impact the number of identified proteins when falling below a certain threshold, primarily due to changes in the amount of injected protein. Of note, while the loss of total protein could be compensated with increased injected material, this solution will not rescue low abundant proteins, primarily due to losses in the sample preparation procedure.

Further, the comparison of sample preparation strategies indicated that with reasonably high cell amount, PMU lysis consistently yielded higher protein amounts and effective release of proteins from all cellular compartments, when compared to RIPA. This discrepancy can likely be ascribed to the precipitation-based methodology utilized by RIPA, which entails higher protein and peptide losses.[188] However, when it comes to one spheroid sample preparation, the freeze-thaw protocol with minimal handling yielded 43 to 69% more proteins being quantified compared to the use of chemical lysis (Paper IV, Figure 3). Lastly, more proteins were quantified using the LF approach rather than TMT.

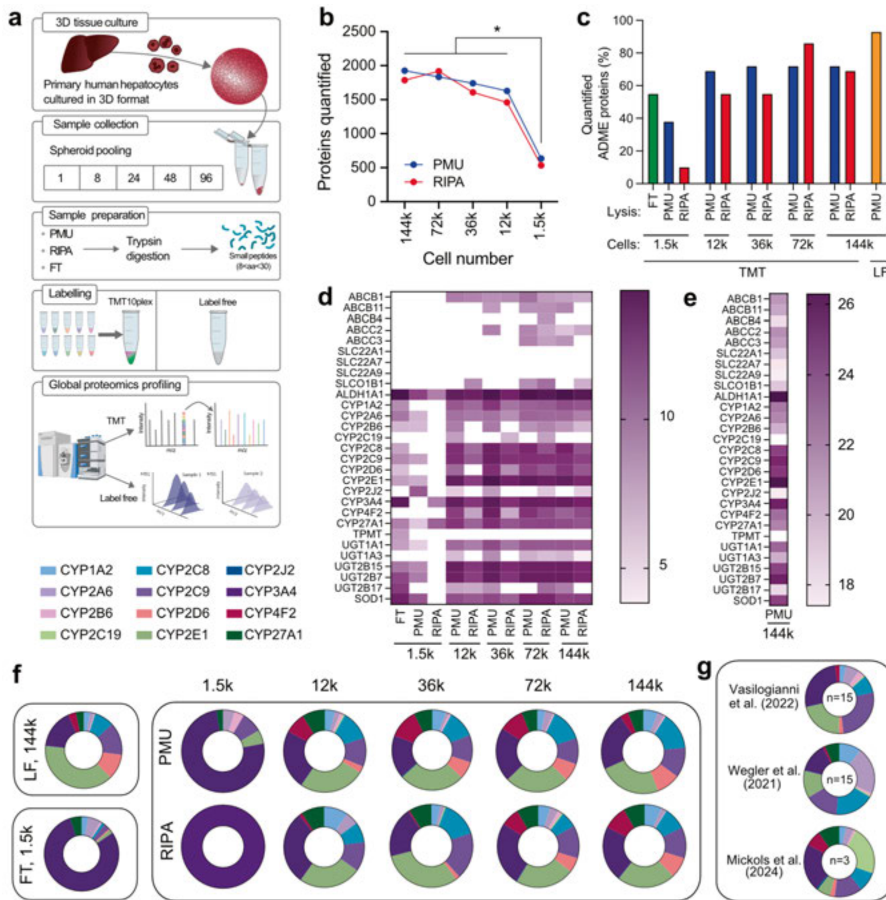

Figure 11. Proteomic workflows for deep phenotypic profiling of 3D organotypic liver models. **a.** Graphical overview summarizing the assessed workflows. The assessed parameters included: initial material amount (1, 8, 24, 48 or 96 3D PHH 1500 PHH each); sample preparation technique using ProteaseMAX (PMU), Radio immunoprecipitation assay buffer (RIPA), or freeze-thaw (FT) method; and quantification strategy based on either tandem mass tags (TMT) or label-free (LF) approach. **b.** The association between protein input and the number of quantified proteins rapidly saturates. **c.** The number of clinically relevant ADMET proteins detected with the different proteomic workflows. **d and e.** Heatmap depicting mean protein abundances (log2 transformed) of clinically relevant ADMET proteins after TMT-based or LF quantification respectively. **f.** Pie charts demonstrating CYP isoform compositions as quantified by the different proteomic workflows. Isoform compositions of representative liver samples are shown as reference. [75,99,189]

For our specific applications, we were most interested whether proteomic workflows impact the quantification of ADMET-related proteins. For that, we compiled a list of 29 clinically important hepatic transporters and DMEs mentioned in the Pharmacogenomic Biomarkers in Drug Labelling guidelines by FDA,[190] and compared their expression in 3D PHH using different proteomic

workflows (Figure 11d,e). We observed a stark difference in the performance of different methods, however the observations followed the same trends as on the global scale — when material is not limited, LF identifies most of the ADMET-proteins (92%), and in limited initial material, PMU drastically outperforms RIPA. Interestingly, we observed that with using only single spheroids, the FT protocol allowed the quantification of more than half of the selected ADMET proteins (16/29). However, none of these proteins belong to ABC and SLC families — quantification of transporters was not possible from single spheroids, regardless of the lysis method. On that note, for transporters quantification gross amount of initial material is essential and LF (all 9 transporters quantified) quantification notably outperforms TMT strategy (only 4 transporters quantified).

Phase I and phase II drug metabolizing enzymes were overall more readily detected than transporters with 75%–95% being quantified for cellular inputs above 12,000 cells (8 spheroids). Impressively, using just one spheroid in FT and PMU sample preparation procedures, we were able to quantify more than half of CYP isoforms. Since CYP isoforms could have been detected by all methods, we compared whether the composition of these isoforms measured with a given method was representative of genuine CYP composition in 3D PHH and liver samples. Interestingly, while CYP3A4 seemed most abundant when analysing single spheroids (Figure 11f), the use of higher cellular inputs indicated that CYP3A4 and CYP2E1 are present at approximately similar amounts. These results align well with isoform compositions identified in the other liver proteomics studies (Figure 11g).[99,189,191] Thus, even if many CYPs could be quantified with a given workflow in one spheroid, the results should be treated with caution. Nonetheless, starting from just eight PHH spheroids, the workflows provided reliable quantifications of CYP isoform composition.

Generally, these findings underscore the importance of the critical assessment of a tool selected for phenotypical description of *in vitro* cultures. Whilst mass-spectrometry based global proteomics provide a unique window into the biology of the selected model, the choice of the workflow, as we demonstrate (here and in the Paper IV), significantly affects the biological interpretation of results, thus should be made with care. In Paper IV we presented standardized and scalable workflows that allow researchers to draw reliable and biologically meaningful conclusions from the analysis of 3D PHH. These results would aid researcher in our field in making an informed decision on the approach to the phenotypical screening and of precious (due to the limited availability of primary cells) PHH cultures. *Interestingly, the method that essentially provided the highest number of quantified proteins and robustness of quantification (at the expense of mass-spec time), was the PMU LF approach that is most similar to what was used and applied in Papers I, II and V.*

Animal product-free 3D PHH (Paper V)

By looking back at the results presented in Papers I-IV, it becomes clear that a significant effort has been put into characterization and optimization of 3D PHH. The main intention behind all that work is to increase the understanding and, ultimately, reproducibility and interpretability of the results obtained when using that *in vitro* culture. Thus, in Paper V, we tackle the last obstacle on the way to fully defined 3D PHH cultures — bovine serum. While other studies indeed eventually achieve chemically defined serum-free 3D PHH cultures, the initial formation of 3D PHH is highly dependent on foetal bovine serum (FBS) supplementation.[12,90,91,99,100,118,160]

The use of FBS is a routine laboratory practice, it has been used for the maintenance of cell cultures for nearly 70 years and remains the most common cell culture medium supplement.[192] FBS is added to the culture medium to facilitate cell attachment and growth, as well as to help the cells circumvent the stress induced by the *in vitro* culture setting. However, the molecular composition of FBS and its effects on cultured cells are prone to batch-to-batch variation and are poorly understood at the molecular level.[193–195] In addition, the use of FBS simply does not adhere to the 3Rs (Refinement, Reduction and Replacement of animal experiments), as FBS production is associated with severe animal welfare issues.[196]

In Paper V we utilized an animal serum-free mixture developed by Rafnsdóttir et al.,[119] and followed Papers I-IV by culturing 3D PHH in Corning 96-well plates in physiologically relevant Williams´ E culture medium, and using label-free mass-spectrometry based global proteomics for phenotypic description of these cultures. Importantly, serum-free media for PHH cultures are not novel and there are a number of life science suppliers offering similar alternatives. However, every single one of these media has undisclosed content. As opposed to proprietary solutions, the approach provided by Rafnsdóttir et al. is fully revealed and well-described, thus opening an opportunity for customization of serum-free medium (SFM) and adaptation of the basal components to the cell culture of choice, in our case to Williams´ E normoglycemic medium for 3D PHH (Figure 12a).

First, we observed that switching from FBS to SFM neither had an impact on the spheroid formation process, nor on 3D PHH viability (Figure 12b). Overall, there was a trend of slightly higher viability in FBS cultures (Figures 11b, e); however, this variation was within well-established limits (80-120% of control values).[14,99,117] These results were interpreted as positive, since we consistently observed when working with 3D PHH, that the absence of FBS or serum substitute when seeding PHH in ULA-plates eventually results in cells not forming spheroids at all (Figure 12c, Donor 1).

Since successful spheroids formation was consistently achieved in all five PHH donors, we randomly selected one of them (Donor 1) and proceeded with evaluating the effect of 3D PHH formation in SFM on long-term cultures.

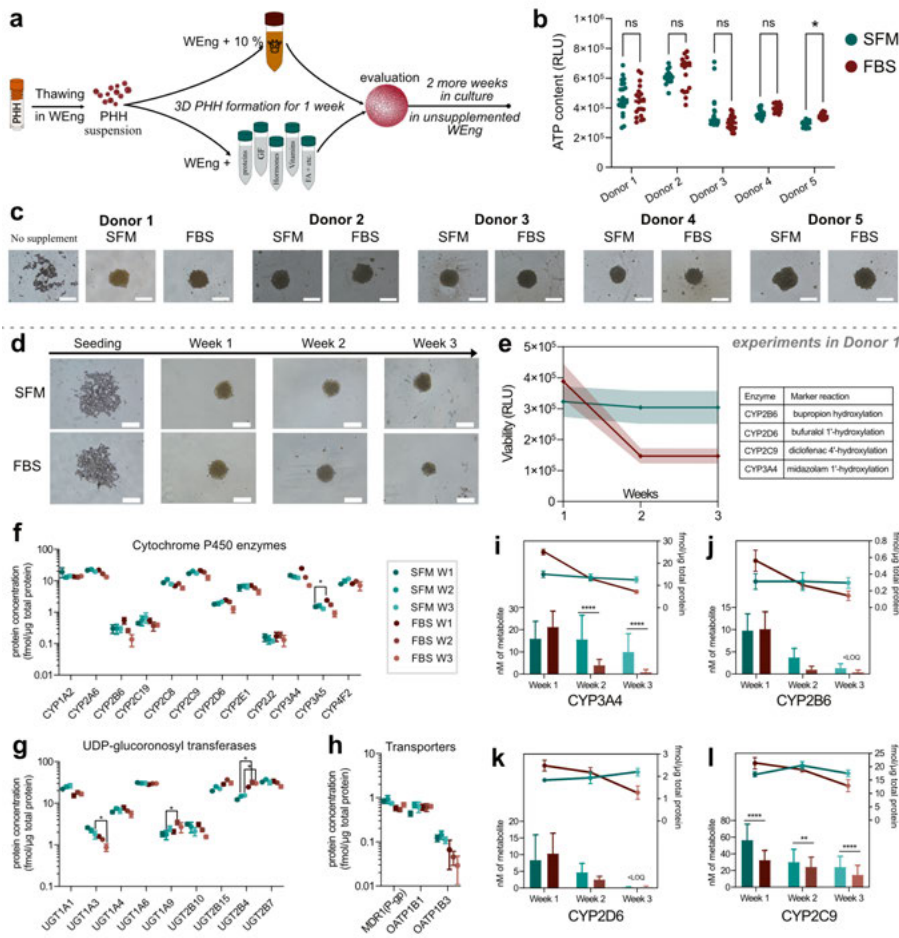

Figure 12. 3D PHH formation and function in conventional FBS supplemented medium or in serum-free medium (SFM). **a.** Schematic overview of the 3D PHH seeding and culture maintenance procedures. PHH were thawed in William´s E normoglycemic medium (WEng) and seeded with 10 % FBS or serum substitute according to Rafnsdottir et al.[119] **b.** Viability (ATP content) of 3D PHH formed in five different donors in SFM or with FBS supplementation. **c.** 3D PHH formation in five donors. Bright-field microscopy images were taken at seeding, after the third day in culture and after one week in culture. Scale bar = 250µm. **d.** 3D PHH spheroid formation in SFM or FBS-supplemented medium and corresponding morphology for three weeks in culture. Scale bar = 250 µm. **e.** Mean viability (ATP content) for three weeks in culture in SFM (green) or FBS-supplemented medium (brown), the shaded area represents standard deviation (n=18-24). **f-h.** Expression levels of quantified cytochrome CYPs, UGTs and drug transporters listed in ICH M12 guidelines. Datapoints in all graphs represent mean expression values of four biological replicates with standard deviation. Stars signify differentially expressed proteins in differential expression analysis (DeqMS). **i-l**. Side-by-side comparison of expression and activity of CYP3A4, CYP2B6, CYP2D6 and CYP2C9 respectively <LOQ signifies metabolites levels below the limit of quantification. *Note experiments presented on panels a-c were performed in five donors, while experiments d-l were performed in Donor 1 only.*

Consistently with previous observation, 3D PHH formed in SFM preserved their morphology for three weeks (Figure 12d). Likewise, we measured viability in these 3D PHH (Figure 12e). Interestingly, whilst ATP measurements were rather comparable between FBS- and SFM-formed spheroids and slightly higher in FBS during the first week of culture, a notable decrease (2-fold) in ATP quantities was seen for spheroids formed in FBS by the second week in culture (Figure 12e). Meanwhile, ATP values remained stable in 3D PHH formed in SFM for the whole duration of culturing. Since FBS is a biological product exhibiting high variability, we could not directly compare its composition to SFM to explain the observed phenomenon. FBS typically contains growth and attachment factors, hormones, nutrients including sugars and lipids, and many other components. The effects of these are inseparable, essentially preventing the interpretation of the obtained results.

To validate the applicability of serum-free 3D PHH for drug disposition studies, we measured protein expression levels of the clinically relevant ADMET proteins listed in the recently released IHC M12 guidelines on drug interaction studies (Figure12f-h).[168] Overall, the expression of ADMET-related proteins across all conditions was stable and very similar between SFM and FBS formed spheroids. We barely found any statistical difference in CYP expression levels, variation in expression of CYP3A5 (Figure 12f) and some of the UGTs (Figure 12g).

Lastly, we compared the expression levels and the obtained activity of CYPs (Figure 12i-l). Similarly to what has been demonstrated before, we observed that in conventional FBS-based conditions, CYP expression and activity had a tendency of decreasing in 3D PHH along the culture time (Figure 12i-l).[12,15,65] However, we noted that 3D PHH formed in SFM exhibited a stable expression of CYPs over three weeks of culturing. In addition, we noted higher and more stable CYP activity in 3D PHH formed in SFM, in particular for CYP3A4 (Figures 12i). As discussed in previous sections, apparent CYP activity derives from a multitude of factors, that could have been affected by a switch from FBS to SFM environment. For instance, as proposed by Kanebratt et al., an observed decrease in CYP activity could be connected to a decrease in ATP content.[15] Alternatively, a switch from FBS to SFM could have affected unbound drug concentration or transporter function.

In summary, our data demonstrates that 3D PHH could be formed in serum-free 3R-compliant chemically defined medium, and successfully used for *in vitro* drug disposition studies. In addition, the use of SFM facilitates the interpretation of research results (more examples in Paper V). Yet, our long-term studies were performed using only one PHH donor, hence the need to be treated with caution until further verification. Lastly, there is an opportunity for further improvements of SFM cell culture medium by substitution of human-derived proteins (e.g. plasma-derived human serum albumin or placenta-derived laminins) to pure recombinant proteins. *And I am looking forward to the developments in this field.*

Conclusions

This thesis provides an improved understanding and optimization of 3D primary human hepatocyte spheroid cultures, and significantly contributes to the harmonization of 3D PHH culture approaches across laboratories and deep integration of this *in vitro* model into drug development pipelines. Finally, a further effort was made to increase 3Rs compliance and standardisation by reducing batch differences, providing a resource for development of serum-free cell culture medium for advanced *in vitro* cultures.

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

- The choice of culture media has a significant effect on the morphology and performance of 3D PHH cultures, and physiologically relevant cell culture medium with fasting levels of glucose and insulin could be successfully used as an alternative to the hyperglycemic media counterparts.

- Normoglycemic William´s E medium can be used for culturing of 3D PHH for drug disposition studies.

- Only certain brands of ultra-low attachment culture plates facilitate the successful 3D PHH spheroid formation, and the choice of the culture plate has a pronounced influence on the 3D PHH performance in drug disposition studies.

- Mass-spectrometry based global proteomics serves as an indispensable tool for phenotypic description of 3D PHH, however the choice of workflow for this analysis has a significant impact on biological interpretation of the results and should be made with caution on a project-to-project basis. Further, with the right protocols, meaningful proteomic fingerprints can be derived from single PHH spheroids. This opens for the possibility of combining scalable microculture with microanalytics.

- Human hepatocyte spheroids could be smoothly formed and cultured in FBS-free conditions, which increases 3Rs compliance, interpretability and applicability of these cultures.

Future perspective

This doctoral thesis contributes to increasing knowledge of 3D PHH culture *per se*. That essentially grants individual researchers more freedom to customise their 3D PHH cultures without compromising on the quality of the spheroid. An ideal 3D PHH is not the one cultured in low glucose and insulin, or serum-free medium, but a spheroid that best serves the needs of the experiment, and, most importantly, permits interpretability of the generated research data. In this thesis and accompanying papers, the following parameters that contribute to the scientific reproducibility of 3D PHH cultures are discussed and evaluated: selection of PHH donor, cell culture medium and its´ composition, FBS supplementation, ULA culture plates and approaches to the phenotypical description of the cultures. Sufficient control over these parameters permits in depth *in vitro* studies of DILI, metabolic liver diseases, liver zonation and physiology, and many more.

As mentioned in the introduction, in last decades the drug development industry is currently making a major leap in substituting traditional *in vivo* animal-based assays with *in vitro* cell cultures. While this doctoral thesis is focused on 3D PHH, the developmental approach and evaluation strategy showcased in the included papers should be translatable to other *in vitro* cultures.

For instance, one of the key parameters to be optimised when developing organ-on-a-chip devices is selecting a cell culture medium that meets nutritional requirements and phenotypic cues of all studied cultures simultaneously.[94] In addition, serum free medium is highly advised for these applications, since FBS constitutes a large source of variability in *in vitro* assays. 3D PHH culture conditions developed and characterised in this thesis serve as an ideal starting point for the development of such universal serum free culture conditions. Further, the presented approach of culturing cells in physiologically relevant, fully humanised and well described medium has a potential to aid in the development of other primary human *in vitro* cultures. The results in this thesis have therefore initiated the development of primary human jejunal and Caco-2 cultures according to the strategies applied for 3D PHH and succeeded in achieving physiologically relevant enteroid and serum-free Caco-2 cultures. Excitingly, in our pilot studies both of these cultures outperform the conventional counterparts. Moreover, it has been demonstrated that the possibility of varying nutritional and hormonal content of the cell culture media, allows accurate modelling of chronic liver disease *in vitro*,[100,161,197] and

culture conditions evaluated in this thesis widen the array of possible experimental set-ups.

That said, adoption of serum-free and/or physiologically relevant medium by both industry and academia shall require significant initial investment in terms of re-designing standard operation procedures, performing in house functional benchmarking of novel cultures and simply ordering and preparing new mixtures and media. Crossing this threshold will most probably be justified in case of struggles with currently used protocol or a need in adjustment of cell culture conditions for specific experiments, but doubtedly otherwise. Nonetheless, in our manuscript we provide detailed summary of the media composition, and using these resources, custom-made media could be prepared whether in house, or ordered from the life science suppliers. On the other hand, the results of the ULA-plates evaluation presented in Paper III seems to be straightforward enough to apply within industry and academia. The stark contrast between the performance of 3D PHH in different ULA plates simply necessitates transition to plate brands that permit proper 3D PHH function.

Furthermore, demonstrate we for the first time that transporter drug-drug interaction studies could successfully be performed in 3D PHH, and hope that this will inspire other drug development stakeholders to adopt physiologically relevant 3D PHH for such studies. In the same study we once again confirm that regulation of drug transport proteins occurs on multiple biological levels,[33] and further studies need to be performed to achieve a mechanistic understanding of this complex process. For instance, regulation of transporters expression via nuclear factors, understanding of the relationship between mRNA and protein abundance, half-life and turn-over rates of drug transporters are of particular interest.[198,199]

Additionally, in this thesis we demonstrate how global proteomics could be routinely used for phenotypic description of 3D PHH cultures. Importantly, in Paper IV we assessed easily available and affordable approaches to perform mass-spectrometry based global proteomics analysis on spheroid cultures. We highlighted the advantages and pitfalls of these approaches, so that researchers within broader field will be able to integrate this molecular technique in their experimental workflows. Since this type of analysis could be purchased from specialised MS facilities, global proteomics analysis being increasingly used for description of cellular phenotypes in drug development. Alas, we acknowledge that subsequent data analysis of protein expression, even in the cases when data is produced by a facility, remains challenging. To address that, we have been working on the development of R-based data analysis software package that allows streamline analysis of global proteomes with particular focus on ADMET-associated proteins. Once published, this workflow will aid in making proteomics analysis a lot easier for non-experts.

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References

1. Pritchard JF, Jurima-Romet M, Reimer MLJ, Mortimer E, Rolfe B, Cayen MN. Making Better Drugs: Decision Gates in Non-Clinical Drug Development. *Nat Rev Drug Discov*. 2003;2(7):542-553. doi:10.1038/nrd1131
2. Pognan F, Beilmann M, Boonen HCM, et al. The evolving role of investigative toxicology in the pharmaceutical industry. *Nat Rev Drug Discov*. 2023;22(4):317-335. doi:10.1038/s41573-022-00633-x
3. Nuwer R. US agency seeks to phase out animal testing. *Nature*. Published online November 4, 2022. doi:10.1038/d41586-022-03569-9
4. FDA no longer needs to require animal tests before human drug trials. Accessed December 26, 2024. https://www.science.org/content/article/fda-no-longer-needs-require-animal-tests-human-drug-trials
5. Soars MG, McGinnity DF, Grime K, Riley RJ. The pivotal role of hepatocytes in drug discovery. *Chemico-Biological Interactions*. 2007;168(1):2-15. doi:10.1016/j.cbi.2006.11.002
6. Ostapowicz G, Fontana RJ, Schiødt FV, et al. Results of a prospective study of acute liver failure at 17 tertiary care centers in the United States. *Ann Intern Med*. 2002;137(12):947-954. doi:10.7326/0003-4819-137-12-200212170-00007
7. Lauschke VM. Toxicogenomics of drug induced liver injury – from mechanistic understanding to early prediction. *Drug Metabolism Reviews*. 2021;53(2):245-252. doi:10.1080/03602532.2021.1894571
8. Weaver RJ, Blomme EA, Chadwick AE, et al. Managing the challenge of drug-induced liver injury: a roadmap for the development and deployment of preclinical predictive models. *Nat Rev Drug Discov*. Published online November 20, 2019:1-18. doi:10.1038/s41573-019-0048-x
9. Naritomi Y, Terashita S, Kagayama A, Sugiyama Y. Utility of hepatocytes in predicting drug metabolism: comparison of hepatic intrinsic clearance in rats and humans in vivo and in vitro. *Drug Metab Dispos*. 2003;31(5):580-588. doi:10.1124/dmd.31.5.580
10. Gerets HHJ, Tilmant K, Gerin B, et al. Characterization of primary human hepatocytes, HepG2 cells, and HepaRG cells at the mRNA level and CYP activity in response to inducers and their predictivity for the detection of human hepatotoxins. *Cell Biol Toxicol*. 2012;28(2):69-87. doi:10.1007/s10565-011-9208-4
11. Lübberstedt M, Müller-Vieira U, Mayer M, et al. HepaRG human hepatic cell line utility as a surrogate for primary human hepatocytes in drug metabolism assessment in vitro. *Journal of Pharmacological and Toxicological Methods*. 2011;63(1):59-68. doi:10.1016/j.vascn.2010.04.013
12. Bell CC, Hendriks DFG, Moro SML, et al. Characterization of primary human hepatocyte spheroids as a model system for drug-induced liver injury, liver function and disease. *Sci Rep*. 2016;6:25187. doi:10.1038/srep25187

13. Dao Thi VL, Wu X, Belote RL, et al. Stem cell-derived polarized hepatocytes. *Nat Commun*. 2020;11(1):1677. doi:10.1038/s41467-020-15337-2
14. Vorrink SU, Zhou Y, Ingelman-Sundberg M, Lauschke VM. Prediction of Drug-Induced Hepatotoxicity Using Long-Term Stable Primary Hepatic 3D Spheroid Cultures in Chemically Defined Conditions. *Toxicol Sci*. 2018;163(2):655-665. doi:10.1093/toxsci/kfy058
15. Kanebratt KP, Janefeldt A, Vilén L, et al. Primary Human Hepatocyte Spheroid Model as a 3D In Vitro Platform for Metabolism Studies. *JPharmSci*. 2021;110(1):422-431. doi:10.1016/j.xphs.2020.10.043
16. Shan J, Schwartz RE, Ross NT, et al. Identification of small molecules for human hepatocyte expansion and iPS differentiation. *Nat Chem Biol*. 2013;9(8):514-520. doi:10.1038/nchembio.1270
17. Ölander M, Wiśniewski JR, Flörkemeier I, Handin N, Urdzik J, Artursson P. A simple approach for restoration of differentiation and function in cryopreserved human hepatocytes. *Arch Toxicol*. 2019;93(3):819-829. doi:10.1007/s00204-018-2375-9
18. Ölander M, Wegler C, Flörkemeier I, et al. Hepatocyte size fractionation allows dissection of human liver zonation. *J Cell Physiol*. 2021;236(8):5885-5894. doi:10.1002/jcp.30273
19. Molina DK, DiMaio VJM. Normal organ weights in men: part II-the brain, lungs, liver, spleen, and kidneys. *Am J Forensic Med Pathol*. 2012;33(4):368-372. doi:10.1097/PAF.0b013e31823d29ad
20. Trefts E, Gannon M, Wasserman DH. The liver. *Current Biology*. 2017;27(21):R1147-R1151. doi:10.1016/j.cub.2017.09.019
21. Boron, W.F. and Boupaep, E.L. (2016) Medical Physiology. 3rd Edition, Elsevier Publisher, Philadelphia.
22. Pouwels S, Sakran N, Graham Y, et al. Non-alcoholic fatty liver disease (NAFLD): a review of pathophysiology, clinical management and effects of weight loss. *BMC Endocrine Disorders*. 2022;22(1):63. doi:10.1186/s12902-022-00980-1
23. Taddei T, Mistry P, Schilsky ML. Inherited metabolic disease of the liver. *Current Opinion in Gastroenterology*. 2008;24(3):278. doi:10.1097/MOG.0b013e3282fcbc0f
24. Manco R, Itzkovitz S. Liver zonation. *J Hepatol*. 2021;74(2):466-468. doi:10.1016/j.jhep.2020.09.003
25. Ding C, Li Y, Guo F, et al. A Cell-type-resolved Liver Proteome. *Mol Cell Proteomics*. 2016;15(10):3190-3202. doi:10.1074/mcp.M116.060145
26. Ölander M, Wiśniewski JR, Artursson P. Cell-type-resolved proteomic analysis of the human liver. *Liver Int*. 2020;40(7):1770-1780. doi:10.1111/liv.14452
27. Nguyen-Lefebvre AT, Horuzsko A. Kupffer Cell Metabolism and Function. *J Enzymol Metab*. 2015;1(1):101.
28. Gissen P, Arias IM. Structural and functional hepatocyte polarity and liver disease. *J Hepatol*. 2015;63(4):1023-1037. doi:10.1016/j.jhep.2015.06.015
29. Ortiz C, Schierwagen R, Schaefer L, Klein S, Trepat X, Trebicka J. Extracellular Matrix Remodeling in Chronic Liver Disease. *Curr Tissue Microenvir Rep*. 2021;2(3):41-52. doi:10.1007/s43152-021-00030-3
30. Klaas M, Kangur T, Viil J, et al. The alterations in the extracellular matrix composition guide the repair of damaged liver tissue. *Sci Rep*. 2016;6(1):27398. doi:10.1038/srep27398

31. McQuitty CE, Williams R, Chokshi S, Urbani L. Immunomodulatory Role of the Extracellular Matrix Within the Liver Disease Microenvironment. *Front Immunol*. 2020;11:574276. doi:10.3389/fimmu.2020.574276
32. Cocucci E, Kim J, Bai Y, Pabla N. Role of Passive Diffusion, Transporters, and Membrane Trafficking-Mediated Processes in Cellular Drug Transport. *Clinical Pharmacology & Therapeutics*. 2017;101(1):121-129. doi:10.1002/cpt.545
33. Galetin A, Brouwer KLR, Tweedie D, et al. Membrane transporters in drug development and as determinants of precision medicine. *Nat Rev Drug Discov*. 2024;23(4):255-280. doi:10.1038/s41573-023-00877-1
34. Molecular Biology of the Cell. Adlibris. Accessed December 27, 2024. https://www.adlibris.com/se/bok/molecular-biology-of-the-cell-9780393884821
35. Casarett & Doull's Toxicology: The Basic Science of Poisons, 9th edition | AccessPharmacy | McGraw Hill Medical. Accessed December 28, 2024. https://accesspharmacy.mhmedical.com/book.aspx?bookid=2462
36. Romeo M, Dallio M, Federico A. Is it time to reconsider the MRP6 role in bile secretion and LPAC genetic signature? *Journal of Hepatology*. 2023;79(4):e152-e154. doi:10.1016/j.jhep.2023.06.012
37. Kwon D, Kim SM, Correia MA. Cytochrome P450 endoplasmic reticulum-associated degradation (ERAD): therapeutic and pathophysiological implications. *Acta Pharm Sin B*. 2020;10(1):42-60. doi:10.1016/j.apsb.2019.11.002
38. Neve EPA, Ingelman-Sundberg M. Cytochrome P450 proteins: retention and distribution from the endoplasmic reticulum. *Curr Opin Drug Discov Devel*. 2010;13(1):78-85.
39. Knockaert L, Fromenty B, Robin MA. Mechanisms of mitochondrial targeting of cytochrome P450 2E1: physiopathological role in liver injury and obesity. *The FEBS Journal*. 2011;278(22):4252-4260. doi:10.1111/j.1742-4658.2011.08357.x
40. Šrejber M, Navrátilová V, Paloncýová M, et al. Membrane-attached mammalian cytochromes P450: An overview of the membrane's effects on structure, drug binding, and interactions with redox partners. *Journal of Inorganic Biochemistry*. 2018;183:117-136. doi:10.1016/j.jinorgbio.2018.03.002
41. Liao BM, McManus SA, Hughes WE, Schmitz-Peiffer C. Flavin-Containing Monooxygenase 3 Reduces Endoplasmic Reticulum Stress in Lipid-Treated Hepatocytes. *Mol Endocrinol*. 2016;30(4):417-428. doi:10.1210/me.2015-1217
42. Radominska-Pandya A, Czernik PJ, Little JM, Battaglia E, Mackenzie PI. Structural and functional studies of UDP-glucuronosyltransferases. *Drug Metab Rev*. 1999;31(4):817-899. doi:10.1081/dmr-100101944
43. Liu Y, Coughtrie MWH. Revisiting the Latency of Uridine Diphosphate-Glucuronosyltransferases (UGTs)—How Does the Endoplasmic Reticulum Membrane Influence Their Function? *Pharmaceutics*. 2017;9(3):32. doi:10.3390/pharmaceutics9030032
44. Human Sulfotransferases and Their Role in Chemical Metabolism | Toxicological Sciences | Oxford Academic. Accessed December 28, 2024. https://academic.oup.com/toxsci/article/90/1/5/1692243
45. Raza H. Dual Localization of Glutathione S-Transferase in the Cytosol and Mitochondria: Implications in Oxidative Stress, Toxicity and Disease. *FEBS J*. 2011;278(22):4243-4251. doi:10.1111/j.1742-4658.2011.08358.x
46. Glutathione transferases: substrates, inihibitors and pro-drugs in cancer and neurodegenerative diseases | Oncogenesis. Accessed December 28, 2024. https://www.nature.com/articles/s41389-017-0025-3

47. Gaweska H, Fitzpatrick PF. Structures and Mechanism of the Monoamine Oxidase Family. *Biomolecular concepts*. 2011;2(5):365. doi:10.1515/BMC.2011.030
48. Nagatsu T. Progress in monoamine oxidase (MAO) research in relation to genetic engineering. *Neurotoxicology*. 2004;25(1-2):11-20. doi:10.1016/S0161-813X(03)00085-8
49. Karlgren M, Bergström CAS. How Physicochemical Properties of Drugs Affect Their Metabolism and Clearance. Published online November 20, 2015. doi:10.1039/9781782622376-00001
50. Zanger UM, Schwab M. Cytochrome P450 enzymes in drug metabolism: regulation of gene expression, enzyme activities, and impact of genetic variation. *Pharmacol Ther*. 2013;138(1):103-141. doi:10.1016/j.pharmthera.2012.12.007
51. Benedetti MS. Biotransformation of xenobiotics by amine oxidases. *Fundam Clin Pharmacol.* 2001 Apr;15(2):75-84. doi: 10.1046/j.1472-8206.2001.00011.x.
52. Basaran R, Can Eke B. Flavin Containing Monooxygenases and Metabolism of Xenobiotics. *Turk J Pharm Sci*. 2017;14(1):90-94. doi:10.4274/tjps.30592
53. Williams JA, Hyland R, Jones BC, et al. Drug-drug interactions for UDP-glucuronosyltransferase substrates: a pharmacokinetic explanation for typically observed low exposure (AUCi/AUC) ratios. *Drug Metab Dispos*. 2004;32(11):1201-1208. doi:10.1124/dmd.104.000794
54. Buratti FM, Darney K, Vichi S, et al. Human variability in glutathione-S-transferase activities, tissue distribution and major polymorphic variants: Meta-analysis and implication for chemical risk assessment. *Toxicology Letters*. 2021;337:78-90. doi:10.1016/j.toxlet.2020.11.007
55. Dubaisi S, Caruso JA, Gaedigk R, et al. Developmental Expression of the Cytosolic Sulfotransferases in Human Liver. *Drug Metab Dispos*. 2019;47(6):592-600. doi:10.1124/dmd.119.086363
56. Riches Z, Stanley EL, Bloomer JC, Coughtrie MWH. Quantitative Evaluation of the Expression and Activity of Five Major Sulfotransferases (SULTs) in Human Tissues: The SULT "Pie." *Drug Metab Dispos*. 2009;37(11):2255-2261. doi:10.1124/dmd.109.028399
57. Xie C, Yan T meng, Chen J mei, et al. LC-MS/MS quantification of sulfotransferases is better than conventional immunogenic methods in determining human liver SULT activities: implication in precision medicine. *Sci Rep*. 2017;7(1):3858. doi:10.1038/s41598-017-04202-w
58. Rotundo L, Pyrsopoulos N. Liver injury induced by paracetamol and challenges associated with intentional and unintentional use. *World Journal of Hepatology*. 2020;12(4):125. doi:10.4254/wjh.v12.i4.125
59. Cox CR, Lynch S, Goldring C, Sharma P. Current Perspective: 3D Spheroid Models Utilizing Human-Based Cells for Investigating Metabolism-Dependent Drug-Induced Liver Injury. *Front Med Technol*. 2020;2. doi:10.3389/fmedt.2020.611913
60. Aleksunes LM, Klaassen CD. Coordinated Regulation of Hepatic Phase I and II Drug-Metabolizing Genes and Transporters using AhR-, CAR-, PXR-, PPARα-, and Nrf2-Null Mice. *Drug Metab Dispos*. 2012;40(7):1366-1379. doi:10.1124/dmd.112.045112
61. Tolson AH, Wang H. Regulation of drug-metabolizing enzymes by xenobiotic receptors: PXR and CAR. *Advanced Drug Delivery Reviews*. 2010;62(13):1238-1249. doi:10.1016/j.addr.2010.08.006
62. Paradiso A, Volpi M, Rinoldi C, et al. In vitro functional models for human liver diseases and drug screening: beyond animal testing. *Biomaterials Science*. 2023;11(9):2988-3015. doi:10.1039/D1BM01872H

63. Kaur S, Kidambi S, Ortega-Ribera M, et al. In Vitro Models for the Study of Liver Biology and Diseases: Advances and Limitations. *Cell Mol Gastroenterol Hepatol*. 2022;15(3):559-571. doi:10.1016/j.jcmgh.2022.11.008
64. Mueller D, Krämer L, Hoffmann E, Klein S, Noor F. 3D organotypic HepaRG cultures as *in vitro* model for acute and repeated dose toxicity studies. *Toxicology in Vitro*. 2014;28(1):104-112. doi:10.1016/j.tiv.2013.06.024
65. Lauschke VM, Hendriks DFG, Bell CC, Andersson TB, Ingelman-Sundberg M. Novel 3D Culture Systems for Studies of Human Liver Function and Assessments of the Hepatotoxicity of Drugs and Drug Candidates. *Chem Res Toxicol*. 2016;29(12):1936-1955. doi:10.1021/acs.chemrestox.6b00150
66. Stanley LA, Wolf CR. Through a glass, darkly? HepaRG and HepG2 cells as models of human phase I drug metabolism. *Drug Metabolism Reviews*. 2022;54(1):46-62. doi:10.1080/03602532.2022.2039688
67. Jennen DGJ, Magkoufopoulou C, Ketelslegers HB, van Herwijnen MHM, Kleinjans JCS, van Delft JHM. Comparison of HepG2 and HepaRG by Whole-Genome Gene Expression Analysis for the Purpose of Chemical Hazard Identification. *Toxicological Sciences*. 2010;115(1):66-79. doi:10.1093/toxsci/kfq026
68. Berger B, Donzelli M, Maseneni S, et al. Comparison Of Liver Cell Models Using The Basel Phenotyping Cocktail. *Frontiers in Pharmacology*. 2016;7. Accessed August 4, 2023. https://www.frontiersin.org/articles/10.3389/fphar.2016.00443
69. Corbett JL, Duncan SA. iPSC-Derived Hepatocytes as a Platform for Disease Modeling and Drug Discovery. *Frontiers in Medicine*. 2019;6. Accessed August 4, 2023. https://www.frontiersin.org/articles/10.3389/fmed.2019.00265
70. Gómez-Lechón MJ, Tolosa L, Conde I, Donato MT. Competency of different cell models to predict human hepatotoxic drugs. *Expert Opin Drug Metab Toxicol*. 2014;10(11):1553-1568. doi:10.1517/17425255.2014.967680
71. Niu L, Geyer PE, Gupta R, et al. Dynamic human liver proteome atlas reveals functional insights into disease pathways. *Mol Syst Biol*. 2022;18(5):e10947. doi:10.15252/msb.202210947
72. Mitaka T. The current status of primary hepatocyte culture. *Int J Exp Pathol*. 1998;79(6):393-409. doi:10.1046/j.1365-2613.1998.00083.x
73. Oliva-Vilarnau N, Vorrink SU, Ingelman-Sundberg M, Lauschke VM. A 3D Cell Culture Model Identifies Wnt/β-Catenin Mediated Inhibition of p53 as a Critical Step during Human Hepatocyte Regeneration. *Adv Sci (Weinh)*. 2020;7(15):2000248. doi:10.1002/advs.202000248
74. Sohlenius-Sternbeck AK, Afzelius L, Prusis P, et al. Evaluation of the human prediction of clearance from hepatocyte and microsome intrinsic clearance for 52 drug compounds. *Xenobiotica*. 2010;40(9):637-649. doi:10.3109/00498254.2010.500407
75. Wegler C, Matsson P, Krogstad V, et al. Influence of Proteome Profiles and Intracellular Drug Exposure on Differences in CYP Activity in Donor-Matched Human Liver Microsomes and Hepatocytes. *Mol Pharm*. 2021;18(4):1792-1805. doi:10.1021/acs.molpharmaceut.1c00053
76. Treyer A, Ullah M, Parrott N, Molitor B, Fowler S, Artursson P. Impact of Intracellular Concentrations on Metabolic Drug-Drug Interaction Studies. *AAPS J*. 2019;21(5):77. doi:10.1208/s12248-019-0344-8
77. Di L, Trapa P, Obach RS, et al. A novel relay method for determining low-clearance values. *Drug Metab Dispos*. 2012;40(9):1860-1865. doi:10.1124/dmd.112.046425

78. Ölander M. *Proteomic and Functional Analysis of In Vitro Systems for Studies of Drug Disposition in the Human Small Intestine and Liver*. 2019. doi:10.13140/RG.2.2.33129.03681
79. Wood FL, Houston JB, Hallifax D. Importance of the Unstirred Water Layer and Hepatocyte Membrane Integrity In Vitro for Quantification of Intrinsic Metabolic Clearance. *Drug Metab Dispos*. 2018;46(3):268-278. doi:10.1124/dmd.117.078949
80. Elaut G, Henkens T, Papeleu P, et al. Molecular mechanisms underlying the dedifferentiation process of isolated hepatocytes and their cultures. *Curr Drug Metab*. 2006;7(6):629-660. doi:10.2174/138920006778017759
81. Xiang C, Du Y, Meng G, et al. Long-term functional maintenance of primary human hepatocytes in vitro. *Science*. 2019;364(6438):399-402. doi:10.1126/science.aau7307
82. Katsuda T, Kawamata M, Inoue A, Yamaguchi T, Abe M, Ochiya T. Long-term maintenance of functional primary human hepatocytes using small molecules. *FEBS Lett*. 2020;594(1):114-125. doi:10.1002/1873-3468.13582
83. Swift B, Pfeifer ND, Brouwer KLR. Sandwich-Cultured Hepatocytes: An In Vitro Model to Evaluate Hepatobiliary Transporter-Based Drug Interactions and Hepatotoxicity. *Drug Metab Rev*. 2010;42(3):446-471. doi:10.3109/03602530903491881
84. Serras AS, Rodrigues JS, Cipriano M, Rodrigues AV, Oliveira NG, Miranda JP. A Critical Perspective on 3D Liver Models for Drug Metabolism and Toxicology Studies. *Frontiers in Cell and Developmental Biology*. 2021;9. Accessed August 4, 2023. https://www.frontiersin.org/articles/10.3389/fcell.2021.626805
85. Zeigerer A, Wuttke A, Marsico G, Seifert S, Kalaidzidis Y, Zerial M. Functional properties of hepatocytes in vitro are correlated with cell polarity maintenance. *Exp Cell Res*. 2017;350(1):242-252. doi:10.1016/j.yexcr.2016.11.027
86. March S, Ramanan V, Trehan K, et al. Micropatterned coculture of primary human hepatocytes and supportive cells for the study of hepatotropic pathogens. *Nat Protoc*. 2015;10(12):2027-2053. doi:10.1038/nprot.2015.128
87. Ware BR, Liu JS, Monckton CP, Ballinger KR, Khetani SR. Micropatterned Coculture With 3T3-J2 Fibroblasts Enhances Hepatic Functions and Drug Screening Utility of HepaRG Cells. *Toxicological Sciences*. 2021;181(1):90-104. doi:10.1093/toxsci/kfab018
88. Cottier KE, Bhalerao D, Lewis C, Gaffney J, Heyward SA. Micropatterned primary hepatocyte co-culture (HEPATOPAC) for fatty liver disease modeling and drug screening. *Sci Rep*. 2023;13(1):15837. doi:10.1038/s41598-023-42785-9
89. Lauschke VM, Shafagh RZ, Hendriks DFG, Ingelman-Sundberg M. 3D Primary Hepatocyte Culture Systems for Analyses of Liver Diseases, Drug Metabolism, and Toxicity: Emerging Culture Paradigms and Applications. *Biotechnology Journal*. 2019;14(7):1800347. doi:10.1002/biot.201800347
90. Bell CC, Dankers ACA, Lauschke VM, et al. Comparison of Hepatic 2D Sandwich Cultures and 3D Spheroids for Long-term Toxicity Applications: A Multicenter Study. *Toxicol Sci*. 2018;162(2):655-666. doi:10.1093/toxsci/kfx289
91. Handin N, Mickols E, Ölander M, et al. Conditions for maintenance of hepatocyte differentiation and function in 3D cultures. *iScience*. 2021;24(11):103235. doi:10.1016/j.isci.2021.103235
92. Preiss LC, Lauschke VM, Georgi K, Petersson C. Multi-Well Array Culture of Primary Human Hepatocyte Spheroids for Clearance Extrapolation of Slowly Metabolized Compounds. *AAPS J*. 2022;24(2):41. doi:10.1208/s12248-022-00689-y

93. Yang S, Ooka M, Margolis RJ, Xia M. Liver three-dimensional cellular models for high-throughput chemical testing. *Cell Reports Methods*. 2023;3(3): 100432. doi:10.1016/j.crmeth.2023.100432
94. A guide to the organ-on-a-chip | *Nature Reviews Methods Primers*. Accessed December 29, 2024. https://www.nature.com/articles/s43586-022-00118-6
95. Allen JW, Khetani SR, Bhatia SN. In vitro zonation and toxicity in a hepatocyte bioreactor. *Toxicol Sci*. 2005;84(1):110-119. doi:10.1093/toxsci/kfi052
96. Wesseler MF, Taebnia N, Harrison S, et al. 3D microperfusion of mesoscale human microphysiological liver models improves functionality and recapitulates hepatic zonation. *Acta Biomaterialia*. 2023;171:336-349. doi:10.1016/j.actbio.2023.09.022
97. Zandi Shafagh R, Youhanna S, Keulen J, et al. Bioengineered Pancreas-Liver Crosstalk in a Microfluidic Coculture Chip Identifies Human Metabolic Response Signatures in Prediabetic Hyperglycemia. *Adv Sci (Weinh)*. 2022;9(34):e2203368. doi:10.1002/advs.202203368
98. Handin N, Mickols E, Ölander M, et al. Conditions for maintenance of hepatocyte differentiation and function in 3D cultures. *iScience*. 2021;24(11):103235. doi:10.1016/j.isci.2021.103235
99. Mickols E, Meyer A, Handin N, et al. OCT1 (SLC22A1) transporter kinetics and regulation in primary human hepatocyte 3D spheroids. *Sci Rep*. 2024;14(1):17334. doi:10.1038/s41598-024-67192-6
100. Youhanna S, Kemas AM, Wright SC, et al. Chemogenomic Screening in a Patient-Derived 3D Fatty Liver Disease Model Reveals the CHRM1-TRPM8 Axis as a Novel Module for Targeted Intervention. *Advanced Science*. n/a(n/a):2407572. doi:10.1002/advs.202407572
101. Bludau I, Aebersold R. Proteomic and interactomic insights into the molecular basis of cell functional diversity. *Nat Rev Mol Cell Biol*. 2020;21(6):327-340. doi:10.1038/s41580-020-0231-2
102. Schwanhäusser B, Busse D, Li N, et al. Global quantification of mammalian gene expression control. *Nature*. 2011;473(7347):337-342. doi:10.1038/nature10098
103. Li JJ, Bickel PJ, Biggin MD. System wide analyses have underestimated protein abundances and the importance of transcription in mammals. *PeerJ*. 2014;2:e270. doi:10.7717/peerj.270
104. Jovanovic M, Rooney MS, Mertins P, et al. Immunogenetics. Dynamic profiling of the protein life cycle in response to pathogens. *Science*. 2015;347(6226):1259038. doi:10.1126/science.1259038
105. Wegler C, Ölander M, Wiśniewski JR, et al. Global variability analysis of mRNA and protein concentrations across and within human tissues. *NAR Genomics and Bioinformatics*. 2020;2(1):lqz010. doi:10.1093/nargab/lqz010
106. Liu Y, Beyer A, Aebersold R. On the Dependency of Cellular Protein Levels on mRNA Abundance. *Cell*. 2016;165(3):535-550. doi:10.1016/j.cell.2016.03.014
107. Meissner F, Geddes-McAlister J, Mann M, Bantscheff M. The emerging role of mass spectrometry-based proteomics in drug discovery. *Nat Rev Drug Discov*. 2022;21(9):637-654. doi:10.1038/s41573-022-00409-3
108. Budayeva HG, Kirkpatrick DS. Monitoring protein communities and their responses to therapeutics. *Nat Rev Drug Discov*. 2020;19(6):414-426. doi:10.1038/s41573-020-0063-y
109. Rosenberger FA, Thielert M, Strauss MT, et al. Spatial single-cell mass spectrometry defines zonation of the hepatocyte proteome. *Nat Methods*. 2023;20(10):1530-1536. doi:10.1038/s41592-023-02007-6

110. Thielert M, Weiss CAM, Mann M, Rosenberger FA. Spatial Proteomics of Single Hepatocytes with Multiplexed Data-Independent Acquisition (mDIA). *Methods Mol Biol*. 2024;2817:97-113. doi:10.1007/978-1-0716-3934-4_9
111. Kurzawa N, Leo IR, Stahl M, et al. Deep thermal profiling for detection of functional proteoform groups. *Nat Chem Biol*. 2023;19(8):962-971. doi:10.1038/s41589-023-01284-8
112. Shuken SR. An Introduction to Mass Spectrometry-Based Proteomics. *J Proteome Res*. 2023;22(7):2151-2171. doi:10.1021/acs.jproteome.2c00838
113. Tyanova, S., Temu, T. & Cox, J. The MaxQuant computational platform for mass spectrometry-based shotgun proteomics. *Nat Protoc* 11, 2301–2319 (2016). https://doi.org/10.1038/nprot.2016.136
114. Barsnes H, Vaudel M. SearchGUI: A Highly Adaptable Common Interface for Proteomics Search and de Novo Engines. *J Proteome Res*. 2018;17(7):2552-2555. doi:10.1021/acs.jproteome.8b00175
115. Palomba A, Abbondio M, Fiorito G, Uzzau S, Pagnozzi D, Tanca A. Comparative Evaluation of MaxQuant and Proteome Discoverer MS1-Based Protein Quantification Tools. *J Proteome Res*. 2021;20(7):3497-3507. doi:10.1021/acs.jproteome.1c00143
116. Lecluyse EL, Alexandre E. Isolation and culture of primary hepatocytes from resected human liver tissue. *Methods Mol Biol*. 2010;640:57-82. doi:10.1007/978-1-60761-688-7_3
117. Xing C, Kemas A, Mickols E, Klein K, Artursson P, Lauschke VM. The choice of ultra-low attachment plates impacts primary human and primary canine hepatocyte spheroid formation, phenotypes, and function. *Biotechnology Journal*. 2024;19(2):2300587. doi:10.1002/biot.202300587
118. Koutsilieri S, Mickols E, Végvári Á, Lauschke VM. Proteomic workflows for deep phenotypic profiling of 3D organotypic liver models. *Biotechnology Journal*. 2024;19(3):2300684. doi:10.1002/biot.202300684
119. Rafnsdóttir ÓB, Kiuru A, Tebäck M, et al. A new animal product free defined medium for 2D and 3D culturing of normal and cancer cells to study cell proliferation and migration as well as dose response to chemical treatment. *Toxicology Reports*. 2023;10:509-520. doi:10.1016/j.toxrep.2023.04.001
120. Bernardi M, Maggioli C, Zaccherini G. Human albumin in the management of complications of liver cirrhosis. *Crit Care.* 2012 Dec 12;16(2):211. doi: 10.1186/cc11218.
121. Sohlenius-Sternbeck AK. Determination of the hepatocellularity number for human, dog, rabbit, rat and mouse livers from protein concentration measurements. *Toxicology in Vitro*. 2006;20(8):1582-1586. doi:10.1016/j.tiv.2006.06.003
122. Sun X, Kaufman PD. Ki-67: more than a proliferation marker. *Chromosoma*. 2018;127(2):175-186. doi:10.1007/s00412-018-0659-8
123. Samali A, Zhivotovsky B, Jones DP, Orrenius S. Detection of pro-caspase-3 in cytosol and mitochondria of various tissues. *FEBS Lett*. 1998;431(2):167-169. doi:10.1016/s0014-5793(98)00740-6
124. Ragnum HB, Vlatkovic L, Lie AK, et al. The tumour hypoxia marker pimonidazole reflects a transcriptional programme associated with aggressive prostate cancer. *Br J Cancer*. 2015;112(2):382-390. doi:10.1038/bjc.2014.604
125. Takahashi Y, Shinoda A, Kamada H, Shimizu M, Inoue J, Sato R. Perilipin2 plays a positive role in adipocytes during lipolysis by escaping proteasomal degradation. *Sci Rep*. 2016;6(1):20975. doi:10.1038/srep20975
126. Rueden CT, Schindelin J, Hiner MC, et al. ImageJ2: ImageJ for the next generation of scientific image data. *BMC Bioinformatics*. 2017;18(1):529. doi:10.1186/s12859-017-1934-z

127. Deroulers C, Ameisen D, Badoual M, Gerin C, Granier A, Lartaud M. Analyzing huge pathology images with open source software. *Diagn Pathol*. 2013;8:92. doi:10.1186/1746-1596-8-92
128. Vildhede A, Karlgren M, Svedberg EK, et al. Hepatic Uptake of Atorvastatin: Influence of Variability in Transporter Expression on Uptake Clearance and Drug-Drug Interactions. *Drug Metab Dispos*. 2014;42(7):1210-1218. doi:10.1124/dmd.113.056309
129. Ahlin G, Chen L, Lazorova L, et al. Genotype-dependent effects of inhibitors of the organic cation transporter, OCT1: predictions of metformin interactions. *Pharmacogenomics J*. 2011;11(6):400-411. doi:10.1038/tpj.2010.54
130. Wiśniewski JR, Mann M. Consecutive proteolytic digestion in an enzyme reactor increases depth of proteomic and phosphoproteomic analysis. *Anal Chem*. 2012;84(6):2631-2637. doi:10.1021/ac300006b
131. Wiśniewski JR, Gaugaz FZ. Fast and sensitive total protein and Peptide assays for proteomic analysis. *Anal Chem*. 2015;87(8):4110-4116. doi:10.1021/ac504689z
132. Hughes, C.S., Moggridge, S., Müller, T. et al. Single-pot, solid-phase-enhanced sample preparation for proteomics experiments. *Nat Protoc* 14, 68–85 (2019). https://doi.org/10.1038/s41596-018-0082-x
133. Perez-Riverol Y, Bandla C, Kundu DJ, Kamatchinathan S, Bai J, Hewapathirana S, John NS, Prakash A, Walzer M, Wang S, Vizcaíno JA. The PRIDE database at 20 years: 2025 update. *Nucleic Acids Res.* 2025 Jan 6;53(D1):D543-D553. doi: 10.1093/nar/gkae1011.
134. Claeys T, Van Den Bossche T, Perez-Riverol Y, Gevaert K, Vizcaíno JA, Martens L. lesSDRF is more: maximizing the value of proteomics data through streamlined metadata annotation. *Nat Commun*. 2023;14(1):6743. doi:10.1038/s41467-023-42543-5
135. Dai C, Füllgrabe A, Pfeuffer J, et al. A proteomics sample metadata representation for multiomics integration and big data analysis. *Nat Commun*. 2021;12(1):5854. doi:10.1038/s41467-021-26111-3
136. Wilkinson, M., Dumontier, M., Aalbersberg, I. et al. The FAIR Guiding Principles for scientific data management and stewardship. *Sci Data* 3, 160018 (2016). https://doi.org/10.1038/sdata.2016.18
137. Cox J, Mann M. MaxQuant enables high peptide identification rates, individualized p.p.b.-range mass accuracies and proteome-wide protein quantification. *Nat Biotechnol*. 2008;26(12):1367-1372. doi:10.1038/nbt.1511
138. Cox J, Neuhauser N, Michalski A, Scheltema RA, Olsen JV, Mann M. Andromeda: a peptide search engine integrated into the MaxQuant environment. *J Proteome Res*. 2011;10(4):1794-1805. doi:10.1021/pr101065j
139. The UniProt Consortium , UniProt: the Universal Protein Knowledgebase in 2025, *Nucleic Acids Research*, Volume 53, Issue D1, 6 January 2025, Pages D609–D617, https://doi.org/10.1093/nar/gkae1010
140. Bielow C, Mastrobuoni G, Kempa S. Proteomics Quality Control: Quality Control Software for MaxQuant Results. *J Proteome Res*. 2016;15(3):777-787. doi:10.1021/acs.jproteome.5b00780
141. Tyanova, S., Temu, T., Sinitcyn, P. et al. The Perseus computational platform for comprehensive analysis of (prote)omics data. *Nat Methods* 13, 731–740 (2016). https://doi.org/10.1038/nmeth.3901
142. Wiśniewski JR. Chapter Four - Label-Free and Standard-Free Absolute Quantitative Proteomics Using the "Total Protein" and "Proteomic Ruler" Approaches. In: Shukla AK, ed. *Methods in Enzymology*. Vol 585. Proteomics in Biology, Part A. Academic Press; 2017:49-60. doi:10.1016/bs.mie.2016.10.002

143. Schröder A, Klein K, Winter S, et al. Genomics of ADME gene expression: mapping expression quantitative trait loci relevant for absorption, distribution, metabolism and excretion of drugs in human liver. *Pharmacogenomics J*. 2013;13(1):12-20. doi:10.1038/tpj.2011.44
144. Thomas PD, Ebert D, Muruganujan A, Mushayahama T, Albou LP, Mi H. PANTHER: Making genome-scale phylogenetics accessible to all. *Protein Science*. 2022;31(1):8-22. doi:10.1002/pro.4218
145. Eden E, Navon R, Steinfeld I, Lipson D, Yakhini Z. GOrilla: a tool for discovery and visualization of enriched GO terms in ranked gene lists. *BMC Bioinformatics*. 2009;10(1):48. doi:10.1186/1471-2105-10-48
146. Rowe C, Gerrard DT, Jenkins R, et al. Proteome-Wide Analyses of Human Hepatocytes During Differentiation and Dedifferentiation. *Hepatology*. 2013;58(2):799. doi:10.1002/hep.26414
147. Dong W, Lu A, Zhao J, Yin S, Ou B, Feng H. An efficient and simple co-culture method for isolating primary human hepatic cells: Potential application for tumor microenvironment research. *Oncology Reports*. 2016;36(4):2126-2134. doi:10.3892/or.2016.4979
148. Forsythe SD, Devarasetty M, Shupe T, et al. Environmental Toxin Screening Using Human-Derived 3D Bioengineered Liver and Cardiac Organoids. *Front Public Health*. 2018;6. doi:10.3389/fpubh.2018.00103
149. Association AD. 1. Improving Care and Promoting Health in Populations: Standards of Medical Care in Diabetes—2021. *Diabetes Care*. 2020;44(Supplement_1):S7-S14. doi:10.2337/dc21-S001
150. Indicator Metadata Registry Details. Accessed January 9, 2025. https://www.who.int/data/gho/indicator-metadata-registry/imr-details/2380
151. Badiu C. Williams Textbook of Endocrinology. *Acta Endocrinol (Buchar)*. 2019;15(3):416. doi:10.4183/aeb.2019.416
152. Grüngreiff K. Zinc in liver disease. *The Journal of Trace Elements in Experimental Medicine*. 2002;15(1):67-78. doi:10.1002/jtra.10002
153. Jarosz M, Olbert M, Wyszogrodzka G, Młyniec K, Librowski T. Antioxidant and anti-inflammatory effects of zinc. Zinc-dependent NF-κB signaling. *Inflammopharmacol*. 2017;25(1):11-24. doi:10.1007/s10787-017-0309-4
154. Arrigoni O, De Tullio MC. Ascorbic acid: much more than just an antioxidant. *Biochim Biophys Acta*. 2002;1569(1-3):1-9. doi:10.1016/s0304-4165(01)00235-5
155. Parker WH, Rhea EM, Qu ZC, Hecker MR, May JM. Intracellular ascorbate tightens the endothelial permeability barrier through Epac1 and the tubulin cytoskeleton. *American Journal of Physiology-Cell Physiology*. 2016;311(4): C652-C662. doi:10.1152/ajpcell.00076.2016
156. Mateus A, Treyer A, Wegler C, Karlgren M, Matsson P, Artursson P. Intracellular drug bioavailability: a new predictor of system dependent drug disposition. *Sci Rep*. 2017;7(1):43047. doi:10.1038/srep43047
157. Kemas AM, Zandi Shafagh R, Taebnia N, et al. Compound Absorption in Polymer Devices Impairs the Translatability of Preclinical Safety Assessments. *Adv Healthc Mater*. 2024;13(11):e2303561. doi:10.1002/adhm.202303561
158. Smith DA, Di L, Kerns EH. The effect of plasma protein binding on in vivo efficacy: misconceptions in drug discovery. *Nat Rev Drug Discov*. 2010;9(12):929-939. doi:10.1038/nrd3287
159. Allonen H, Ziegler G, Klotz U. Midazolam kinetics. *Clinical Pharmacology & Therapeutics*. 1981;30(5):653-661. doi:10.1038/clpt.1981.217
160. Oliva-Vilarnau N, Vorrink SU, Büttner FA, et al. Comparative analysis of YAP/TEAD inhibitors in 2D and 3D cultures of primary human hepatocytes

reveals a novel non-canonical mechanism of CYP induction. *Biochemical Pharmacology*. 2023;215:115755. doi:10.1016/j.bcp.2023.115755
161. Kozyra M, Johansson I, Nordling Å, Ullah S, Lauschke VM, Ingelman-Sundberg M. Human hepatic 3D spheroids as a model for steatosis and insulin resistance. *Sci Rep*. 2018;8. doi:10.1038/s41598-018-32722-6
162. Wang DS, Kusuhara H, Kato Y, Jonker JW, Schinkel AH, Sugiyama Y. Involvement of Organic Cation Transporter 1 in the Lactic Acidosis Caused by Metformin. *Mol Pharmacol*. 2003;63(4):844-848. doi:10.1124/mol.63.4.844
163. White DL, Saunders VA, Dang P, et al. OCT-1–mediated influx is a key determinant of the intracellular uptake of imatinib but not nilotinib (AMN107): reduced OCT-1 activity is the cause of low in vitro sensitivity to imatinib. *Blood*. 2006;108(2):697-704. doi:10.1182/blood-2005-11-4687
164. Zhang S, Lovejoy KS, Shima JE, et al. Organic Cation Transporters Are Determinants of Oxaliplatin Cytotoxicity. *Cancer Research*. 2006;66(17):8847-8857. doi:10.1158/0008-5472.CAN-06-0769
165. Nies AT, Koepsell H, Damme K, Schwab M. Organic Cation Transporters (OCTs, MATEs), In Vitro and In Vivo Evidence for the Importance in Drug Therapy. In: Fromm MF, Kim RB, eds. *Drug Transporters*. Handbook of Experimental Pharmacology. Springer; 2011:105-167. doi:10.1007/978-3-642-14541-4_3
166. Haberkorn B, Fromm MF, König J. Transport of Drugs and Endogenous Compounds Mediated by Human OCT1: Studies in Single- and Double-Transfected Cell Models. *Frontiers in Pharmacology*. 2021;12. Accessed September 6, 2023. https://www.frontiersin.org/articles/10.3389/fphar.2021.662535
167. Chen L, Shu Y, Liang X, et al. OCT1 is a high-capacity thiamine transporter that regulates hepatic steatosis and is a target of metformin. *Proc Natl Acad Sci U S A*. 2014;111(27):9983-9988. doi:10.1073/pnas.1314939111
168. ICH M12 Guideline on drug interaction studies. 2024
169. Jinakote M, Ontawong A, Soodvilai S, et al. High affinity of 4-(4-(dimethylamino)styryl)-N-methylpyridinium transport for assessing organic cation drugs in hepatocellular carcinoma cells. *Fundamental & Clinical Pharmacology*. 2020;34(3):365-379. doi:10.1111/fcp.12531
170. Fattah S, Shinde AB, Matic M, et al. Inter-Subject Variability in OCT1 Activity in 27 Batches of Cryopreserved Human Hepatocytes and Association with OCT1 mRNA Expression and Genotype. *Pharm Res*. 2017;34(6):1309-1319. doi:10.1007/s11095-017-2148-9
171. Morozova GI, Dobretsov GE, Dubur GI, Dubur RR, Golitsyn VM. [4-(n-Dimethylaminostyryl)-1-methylpyridinium fluorescence in a living cell]. *Tsitologiia*. 1981;23(8):916-923.
172. Ahlin G, Karlsson J, Pedersen JM, et al. Structural Requirements for Drug Inhibition of the Liver Specific Human Organic Cation Transport Protein 1. *J Med Chem*. 2008;51(19):5932-5942. doi:10.1021/jm8003152
173. Gore E, Bigaeva E, Oldenburger A, et al. Investigating fibrosis and inflammation in an ex vivo NASH murine model. *American Journal of Physiology-Gastrointestinal and Liver Physiology*. 2020;318(2):G336-G351. doi:10.1152/ajpgi.00209.2019
174. Choudhary NS, Kumar N, Duseja A. Peroxisome Proliferator-Activated Receptors and Their Agonists in Nonalcoholic Fatty Liver Disease. *J Clin Exp Hepatol*. 2019;9(6):731-739. doi:10.1016/j.jceh.2019.06.004
175. Fougerat A, Montagner A, Loiseau N, Guillou H, Wahli W. Peroxisome Proliferator-Activated Receptors and Their Novel Ligands as Candidates for the

Treatment of Non-Alcoholic Fatty Liver Disease. *Cells*. 2020;9(7):1638. doi:10.3390/cells9071638
176. Hirsch C, Schildknecht S. In Vitro Research Reproducibility: Keeping Up High Standards. *Front Pharmacol*. 2019;10:1484. doi:10.3389/fphar.2019.01484
177. Baker M. 1,500 scientists lift the lid on reproducibility. *Nature*. 2016;533(7604):452-454. doi:10.1038/533452a
178. Costa EC, de Melo-Diogo D, Moreira AF, Carvalho MP, Correia IJ. Spheroids Formation on Non-Adhesive Surfaces by Liquid Overlay Technique: Considerations and Practical Approaches. *Biotechnol J*. 2018;13(1). doi:10.1002/biot.201700417
179. Tsai YA, Li T, Torres-Fernández LA, Weise SC, Kolanus W, Takeoka S. Ultra-Thin Porous PDLLA Films Promote Generation, Maintenance, and Viability of Stem Cell Spheroids. *Front Bioeng Biotechnol*. 2021;9. doi:10.3389/fbioe.2021.674384
180. Chou SF, Lai JY, Cho CH, Lee CH. Relationships between surface roughness/stiffness of chitosan coatings and fabrication of corneal keratocyte spheroids: Effect of degree of deacetylation. *Colloids Surf B Biointerfaces*. 2016 Jun 1;142:105-113. doi: 10.1016/j.colsurfb.2016.02.051.
181. Palmgrén JJ, Mönkkönen J, Korjamo T, Hassinen A, Auriola S. Drug adsorption to plastic containers and retention of drugs in cultured cells under in vitro conditions. *Eur J Pharm Biopharm*. 2006;64(3):369-378. doi:10.1016/j.ejpb.2006.06.005
182. Lou R, Cao Y, Li S, et al. Benchmarking commonly used software suites and analysis workflows for DIA proteomics and phosphoproteomics. *Nat Commun*. 2023;14(1):94. doi:10.1038/s41467-022-35740-1
183. Ramus C, Hovasse A, Marcellin M, et al. Benchmarking quantitative label-free LC-MS data processing workflows using a complex spiked proteomic standard dataset. *J Proteomics*. 2016;132:51-62. doi:10.1016/j.jprot.2015.11.011
184. Raffield LM, Dang H, Pratte KA, et al. Comparison of Proteomic Assessment Methods in Multiple Cohort Studies. *Proteomics*. 2020;20(12):e1900278. doi:10.1002/pmic.201900278
185. Zheng W, Yang P, Sun C, Zhang Y. Comprehensive comparison of sample preparation workflows for proteomics. *Mol Omics*. 2022;18(6):555-567. doi:10.1039/D2MO00076H
186. Mund A, Brunner AD, Mann M. Unbiased spatial proteomics with single-cell resolution in tissues. *Molecular Cell*. 2022;82(12):2335-2349. doi:10.1016/j.molcel.2022.05.022
187. Messner S, Fredriksson L, Lauschke VM, et al. Transcriptomic, Proteomic, and Functional Long-Term Characterization of Multicellular Three-Dimensional Human Liver Microtissues. *Appl In Vitro Toxicol*. 2018;4(1):1-12. doi:10.1089/aivt.2017.0022
188. Ngoka LC. Sample prep for proteomics of breast cancer: proteomics and gene ontology reveal dramatic differences in protein solubilization preferences of radioimmunoprecipitation assay and urea lysis buffers. *Proteome Sci*. 2008 Oct 24;6:30. doi: 10.1186/1477-5956-6-30.
189. Vasilogianni AM, Al-Majdoub ZM, Achour B, Annie Peters S, Barber J, Rostami-Hodjegan A. Quantitative Proteomics of Hepatic Drug-Metabolizing Enzymes and Transporters in Patients With Colorectal Cancer Metastasis. *Clin Pharmacol Ther*. 2022;112(3):699-710. doi:10.1002/cpt.2633
190. Table of Pharmacogenomic Biomarkers in Drug Labeling. *FDA*. Published online September 23, 2024. https://www.fda.gov/drugs/science-and-research-drugs/table-pharmacogenomic-biomarkers-drug-labeling

191. Wegler C, Garcia LP, Klinting S, et al. Proteomics-Informed Prediction of Rosuvastatin Plasma Profiles in Patients With a Wide Range of Body Weight. *Clinical Pharmacology and Therapeutics*. 2021;109(3):762. doi:10.1002/cpt.2056
192. Puck TT, Cieciura SJ, Robinson A. Genetics of somatic mammalian cells. III. Long-term cultivation of euploid cells from human and animal subjects. *J Exp Med*. 1958;108(6):945-956. doi:10.1084/jem.108.6.945
193. Gstraunthaler G, Lindl T, van der Valk J. A plea to reduce or replace fetal bovine serum in cell culture media. *Cytotechnology*. 2013;65(5):791-793. doi:10.1007/s10616-013-9633-8
194. Liu S, Yang W, Li Y, Sun C. Fetal bovine serum, an important factor affecting the reproducibility of cell experiments. *Sci Rep*. 2023;13(1):1942. doi:10.1038/s41598-023-29060-7
195. Baker M. Reproducibility: Respect your cells! *Nature*. 2016;537(7620):433-435. doi:10.1038/537433a
196. Valk J van der, Bieback K, Buta C, et al. Fetal bovine serum (FBS): Past – present – future. *ALTEX - Alternatives to animal experimentation*. 2018;35(1):99-118. doi:10.14573/altex.1705101
197. Kralj T, Khatri R, Brouwer KR, Brouwer KLR, Creek DJ. Lipidomics profiles in hepatocytes from nonalcoholic steatohepatitis patients differ markedly from in vitro-induced steatotic hepatocytes. *FEBS Letters*. 2022;596(11):1445-1452. doi:10.1002/1873-3468.14318
198. Czuba LC, Hillgren KM, Swaan PW. Post-translational Modifications of Transporters. *Pharmacol Ther*. 2018;192:88-99. doi:10.1016/j.pharmthera.2018.06.013
199. Brouwer KLR, Evers R, Hayden E, et al. Regulation of Drug Transport Proteins—From Mechanisms to Clinical Impact: A White Paper on Behalf of the International Transporter Consortium. *Clinical Pharmacology & Therapeutics*. 2022;112(3):461-484. doi:10.1002/cpt.2605

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