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MODELLING AND GENETIC CORRECTION OF LIVER GENETIC DISEASES

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MODELLING AND GENETIC CORRECTION OF LIVER GENETIC DISEASES

THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

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Στο Φίλιππο και Χρήστο,

.

“What was inconceivable yesterday,
and barely achievable today,
often becomes routine tomorrow.”

Thomas Starzl

POPULAR SCIENCE SUMMARY OF THE THESIS

The urea cycle is a part of the body's metabolism that converts highly toxic compounds into non-toxic ones and is predominantly present in the liver. Abnormalities in the genes responsible for the synthesis of the cycle's proteins might lead to its dysfunction and life-threatening conditions due to the accumulation of toxic waste. The only definitive treatment for urea cycle defects (UCD) is liver transplantation. However, the scarcity of available organ donors dictates the need for the investigation of alternative treatments. Before new therapeutics are used in patients, they need to be tested and evaluated pre-clinically; on cells in the lab, as well as on experimental animals that exhibit symptoms of the disease similar to those present in affected patients. Platforms which are commonly called "disease models". Overarching aims of this thesis were to investigate potential disease models for the study of UCD, as well as to explore the possibilities of correcting disease-causing DNA defects.

Stem cells can theoretically be converted into any cell type of our body, including liver cells. Currently, the generation of "artificial" stem cells from any individual is possible, but the procedure is sometimes considered inefficient and challenging. On that account, in **PAPER I**, we optimized the current protocol with the introduction of modifications achieving higher efficiency in generating individual's stem cells. Furthermore, when stem cells are differentiated into liver cells, they need to be evaluated to which extent they resemble the authentic liver cells in our bodies; samples that most laboratories do not have access to. To this end, in **PAPER II,** we profiled the gene expression in authentic fetal and adult liver tissues which can serve as an assessment tool for investigators to compare liver cells generated in their laboratories from stem cell sources. Furthermore, in **PAPER III** we provide evidence that lab-made liver cells from patients with UCD display metabolic impairment which is improved after correction of the faulty gene with a technique that "cuts and fixes" defects in the genetic code. In conclusion, **PAPER III** demonstrates that stem cell-derived liver cells could potentially be used as models of UCD, and perhaps serve as a source of patient's own healthy, genetically-corrected cells in the future.

After promising therapeutics having been tested on cells, and before being used in patients, they frequently need to be assessed on laboratory animals. Satisfactory disease animal models present symptoms of the disease similar to the manifestations in human patients. To this end, we used special mice that can be transplanted with defective human liver cells that gradually replace the murine cells in the liver and ultimately, obtain a liver in the mouse that consists almost entirely of human cells. In **PAPERS IV** and **V**, we transplanted these mice with cells from patients with two different UCD. In both cases, the liver-humanized mice displayed symptoms of the diseases indicating that the model can prominently be used to test potential therapeutics targeting these specific disorders. On the contrary, when mice were transplanted with cells in which the faulty gene was corrected applying the "genetic scissors"-technique (CRISPR), the animals were nearly healthy, pointing out the efficacy of genetic correction (**PAPER V**).

In conclusion, the research work conducted in this thesis demonstrates the prospects that the "artificial" stem cells and humanized mice possess for the generation of models of liver genetic diseases. Furthermore, the emergence of genome editing technologies further enhances the aforementioned potentials, as well as raises hopes for the treatment of liver genetic defects through genetic manipulation.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Ureacykeln utgör den metaboliska process i kroppen som omvandlar ammoniak till urea. Denna omvandling av giftiga föreningar till icke-toxiska sker övervägande i levern. Genetiska förändringar associerade till ureacykeln kan bland annat ge upphov till icke-funktionella för processen viktiga proteiner när dessa gener translateras. Detta kan i sin tur leda till en ansamling av gifter som i värsta fall kan resultera i livshotande tillstånd. För närvarande är levertransplantation den enda definitiva behandlingen för ureacykeldefekter (UCD). Bristen på tillgängliga organdonatorer innebär dock att det är ett stort behov av forskning relaterat till alternativa behandlingar. Innan en sådan alternativ terapi kan appliceras på patienter måste dock dess effekt och säkerhet först undersökas rigoröst. I detta ingår bland annat att terapin testas på olika ”sjukdomsmodeller”, vilket innebär celler i laboratoriet eller försöksdjur som uppvisar symtom på sjukdomen som liknar dem som finns hos drabbade patienter. De övergripande målen med denna avhandling var att undersöka potentiella sjukdomsmodeller för studier av UCD, samt att undersöka möjligheterna att korrigera sjukdomsframkallande DNA-avvikelser.

Stamceller kan teoretiskt differentieras till vilken celltyp som helst i vår kropp inklusive leverceller. För närvarande är det möjligt att generera ”artificiella” stamceller från alla individer, men proceduren anses ibland vara ineffektiv och utmanande. Därför optimerade vi, i **PAPER I**, det nuvarande protokollet för att uppnå högre effektivitet för att generera individens stamceller. När stamceller differentieras till leverceller måste de utvärderas i vilken utsträckning de liknar de autentiska levercellerna, vilket är något som de flesta laboratorier inte har möjlighet till. För detta ändamål, i **PAPER II**, karaktäriserade vi genuttrycket i vävnadsprover utvunna från levern i foster och vuxna människor. Detta kan i sin tur fungera som ett bedömningsverktyg för forskare som vill jämföra levercellerna genererade i deras laboratorium från stamcellskällor. Vidare ger vi i **PAPER III** bevis för att laboratorietillverkade leverceller från patienter med UCD uppvisar ureacykeldysfunktion. Denna dysfunktion kunde vi sedan behandla med hjälp av genetisk korrigering av den trasiga genen med hjälp av CRISPR; en teknik som kan ”skära och laga” defekter i den genetiska koden. Sammanfattningsvis visar **PAPER III** att leverceller som härrör från stamceller kan användas som modeller för UCD, liksom en potentiell framtida källa till patientens egna friska, genkorrigerade celler.

Efter att lovande läkemedel undersökts på celler behöver de i regel även valideras i laboratoriedjur innan behandling av patienter. Bra sjukdomsmodeller uppvisar samma typ av sjukdomssymtom som de kliniska manifestationerna hos mänskliga patienter. Vi använde därför speciella möss som kan transplanteras med defekta humana leverceller, vilka gradvis ersätter de murina cellerna i levern till dess att mössens lever består av nästan enbart mänskliga celler. I **PAPER IV** och **V** transplanterade vi dessa möss med leverceller från patienter med två olika UCD. I båda fallen uppvisade de leverhumaniserade mössen symtom på sjukdomarna som indikerar att modellen framträdande kan användas för att testa potentiella terapier inriktade på dessa specifika störningar. När mössen däremot transplanterades med celler i vilka den felaktiga genen korrigerades med CRISPR teknik var mössen istället nästan helt friska, vilket ger prov på möjligheterna med genetisk korrigering (**PAPER V**).

Sammanfattningsvis visar forskningsarbetet i denna avhandling de möjligheter de här ”artificiella” stamcellerna och humaniserade mössen har när det kommer till att generera

modeller för genetiska leversjukdomar. Utöver detta så har utvecklingen av genomredigeringsteknologier ytterligare förbättrat dessa möjligheter, vilket även väcker förhoppningar om behandling av genetiska leversjukdomar genom genetisk manipulation.

SCIENTIFIC ABSTRACT

The urea cycle is a set of biochemical reactions that converts highly toxic ammonia into urea for excretion. Deficiencies in any of the genes of the cycle can be life-threatening, with liver transplantation currently being the only definitive treatment. However, the scarcity of donor organs dictates the investigation of alternative treatments, which requires appropriate disease models, *in vitro* and *in vivo,* that faithfully recapitulate the disease pathology. Recent advancements in the field of genome engineering make interventions in the genetic code less challenging, thereby assisting in the generation of such tools, as well as raising the potential for genetic correction of these conditions. The research conducted in this thesis centres around two broad aims: the investigation of disease models and genetic correction of inherited liver disorders.

Induced pluripotent stem cells (iPSC) hold great potential both for disease modelling and as a source of cells for cell therapy. However, their generation through cell reprogramming is sometimes challenging and inefficient. Therefore, in **PAPER I** we sought to optimize the reprogramming procedure by introducing modifications to the currently existing protocols, and managed to increase the reprogramming efficiency. IPSC could theoretically differentiate into any cell type, including hepatocytes. In order to assess the level of differentiation of the hepatocyte-like cells (HLC) generated from stem cell sources, comparisons with authentic primary liver tissues are necessary. To this end, in **PAPER II** we created gene expression profiles of fetal and mature (post-natal) liver tissues from a significant number of individuals. The dataset can serve as an accurate and simple assessment tool to evaluate and compare HLC, generated in different laboratories, to authentic human liver tissues. If HLC resemble the functions observed in mature primary hepatocytes, they could be used as *in vitro* disease models. In addition, programmable nucleases can be applied to either correct or introduce disease-causing of interest in the genome. In **PAPER III**, we generated iPSC from a patient with a pathogenic variant in the ornithine transcarbamylase (*OTC*) gene, the most common UCD, corrected the genetic defect and differentiated the cells into HLC. The correction was molecularly, as well as phenotypically confirmed by the restoration of urea cycle function.

The thesis also focuses on the investigation of *in vivo* disease models of UCD. Specifically, in **PAPERS IV** and **V** we created liver-humanized mice with hepatocytes from patients with UCD, OTC deficiency (OTCD) or carbamoyl phosphate synthetase 1 deficiency (CPS1D). Highly repopulated animals faithfully recapitulated the clinical manifestations of the disease observed in patients, including hyperammonemia which is considered a hallmark of these UCD. Furthermore, in **PAPER V**, we investigated the efficacy and safety of *ex vivo* gene editing of primary OTCD hepatocytes. Ureagenesis was restored *in vitro* in edited cells, as well as *in vivo* as mice liver-repopulated with genetically engineered cells partially or completely reversed all markers of the disease investigated. Finally, extensive gene expression and deep sequencing analysis revealed no unspecific mutagenesis effected by the programmable nucleases, pointing out the safety of the application.

In conclusion, the research work conducted in this thesis demonstrates the prospects that iPSC and humanized mice possess for the generation of models of liver genetic diseases, *in vitro* and *in vivo*. Furthermore, the emergence of genome editing technologies further enhances the aforementioned potentials, as well as raises possibilities for the treatment of liver genetic defects through genome manipulation.

LIST OF SCIENTIFIC PAPERS

Constituent peer-reviewed publications of this thesis are listed below, and referred to in the text with their roman numerals.

I. Vosough, M., F. Ravaioli, **M. Zabulica**, M. Capri, P. Garagnani, C. Franceschi, J. Piccand, M. R. Kraus, K. Kannisto, R. Gramignoli and S. C. Strom (2019). *Applying hydrodynamic pressure to efficiently generate induced pluripotent stem cells via reprogramming of centenarian skin fibroblasts.* PLoS One 14(4): e0215490.

II. **Zabulica, M.**, R. C. Srinivasan, M. Vosough, C. Hammarstedt, T. Wu, R. Gramignoli, E. Ellis, K. Kannisto, A. Collin de l'Hortet, K. Takeishi, A. Soto-Gutierrez and S. C. Strom (2019). *Guide to the assessment of mature liver gene expression in stem cell-derived hepatocytes.* Stem Cells Dev 28(14): 907-919.

III. **Zabulica, M.**, Jakobsson, T., Ravaioli, F., Vosough, M., Gramignoli, R., Ellis, E., Rooyackers, O., Strom, S. C. (2021) *Gene editing correction of a urea cycle defect in organoid stem cell derived hepatocytes*. Int J. Mol. Sci. 22(3): 1217.

IV. Srinivasan, R. C., **M. Zabulica**, C. Hammarstedt, T. Wu, R. Gramignoli, K. Kannisto, E. Ellis, A. Karadagi, R. Fingerhut, G. Allegri, V. Rufenacht, B. Thony, J. Haberle, J. M. Nuoffer and S. C. Strom (2019). *A liver-humanized mouse model of carbamoyl phosphate synthetase 1-deficiency*. J Inherit Metab Dis 42(6): 1054-1063.

V. **Zabulica, M.**, R. C. Srinivasan, P. Akcakaya, G. Allegri, B. Bestas, M. Firth, C. Hammarstedt, T. Jakobsson, T. Jakobsson, E. Ellis, C. Jorns, G. Makris, T. Scherer, N. Rimann, N. R. van Zuydam, R. Gramignoli, A. Forslow, S. Engberg, M. Maresca, O. Rooyackers, B. Thony, J. Haberle, B. Rosen and S. C. Strom (2021). *Correction of a urea cycle defect after ex vivo gene editing of human hepatocytes*. Mol Ther. 20:S1525-0016(21)00024-1.

CONTENTS

LIST OF ABBREVIATIONS

A1AT	Alpha-1 antitrypsin
AAV	Adeno-associated virus
ABE	Adenine base editor
AFP	Alpha-fetoprotein
Alb	Albumin
ALP	Alkaline phosphatase
apoB	Apolipoprotein B
ARG1	Arginase 1
ASL	Argininosuccinate lyase
ASS1	Argininosuccinate synthetase 1
Cas9	CRISPR-associated 9 nuclease
CBE	Cytosine base editor
COVID	Corona virus disease
CPS1	Carbamoyl phosphate synthetase 1
CRISPR	Clustered regularly interspaced short palindromic repeats
crRNA	CRISPR RNA
ELISA	Enzyme-linked immunosorbent assay
ESC	Embryonic stem cells
FACS	Fluorescence-activated cell sorting
FAH	Fumaryloacetoacetate hydrolase
GLUL	Glutamate-ammonia ligase
GWAS	Genome-wide association study
HBV	Hepatitis B virus
HDR	Homology-directed repair
HLC	Hepatocyte-like cells
HPDD	Hydroxyphenylpyruvate dioxygenase
HT	Hereditary tyrosinemia
hTERT	Human telomerase reverse transcriptase
HTx	Hepatocyte transplantation
ICC	Immunocytochemistry
IEM	Inborn errors of metabolism
IHC	Immunohistochemistry
Il2rg	Interleukin-2 receptor subunit gamma
iPSC	Induced pluripotent stem cells
LDL	Low-density lipoprotein
NAGS	N-acetyl glutamate synthetase
NHEJ	Non-homologous end joining
NOD	Non-obese diabetic
NTBC	(2-(2-nitro-4-trifluoromethylbenzoyl)-1,3-cyclohexanedione)

ORG HLC	Organoid hepatocyte-like cells
ORNT1	Ornithine translocase
OTC	Ornithine transcarbamylase
OTCD	Ornithine transcarbamylase deficient/deficiency
OTCP	Ornithine transcarbamylase proficient
PAH	Phenylalanine hydroxylase
PAM	Protospacer adjacent motif
PCSK9	Proprotein convertase subtilisin/kexin type 9
PKU	Phenylketonuria
PPIA	Peptidylprolyl isomerase A (Cyclophilin A)
SCID	Severe combined immunodeficiency
SNP	Single-nucleotide polymorphism
TALEN	Transcription activator-like effector nucleases
tracrRNA	trans-activating crRNA
TTR	Transthyretin
UCD	Urea cycle disorder
uPA	Urokinase type plasminogen activator
ZFN	Zinc finger nuclease

1 INTRODUCTION

1.1 LIVER

1.1.1 Liver anatomy and organization

Liver is one of the largest organs in the human body. It is located below the diaphragm on the left site of the abdominal pelvic region. It consists of a spongy-like mass of around 1,300-1,700 g, depending on body size and sex, and compromises 2-5% of the total body mass in humans. In order to maintain homeostasis, liver performs a wide range of functions, among others synthesis and elimination of various molecules, metabolism of nutrients and xenobiotics, secretion and storage of metabolic products. The liver consists of seven different cell types – hepatocytes, sinusoidal endothelial cells, macrophages, cholangiocytes, lymphocytes, dendritic and stellate cells. They are organized in an extracellular matrix that mediates the interaction between them and their diverse functions (Table 1).
The main architectural units of the liver are the lobules which are structures that resemble hexagonal plates of hepatocytes with a portal triad consisting of portal vein, hepatic artery, and bile duct, present at each corner (Figure 1). Hepatic histologic analysis reveals a homogenous landscape and blending of the above cells, infiltrated by blood vessels and bile ducts. There are two blood sources perfusing the liver. One is venous blood at low pressure after circulation through the gut, spleen, and pancreas, and it is rich in nutrients, toxins, hormones and growth factors, but low in oxygen. The other is arterial blood with higher oxygen levels and nutrients at normal physiological concentrations and pressure. Portal flow is approximately 70% of the total liver blood flow, and the arterial flow contributes the remaining 30%.

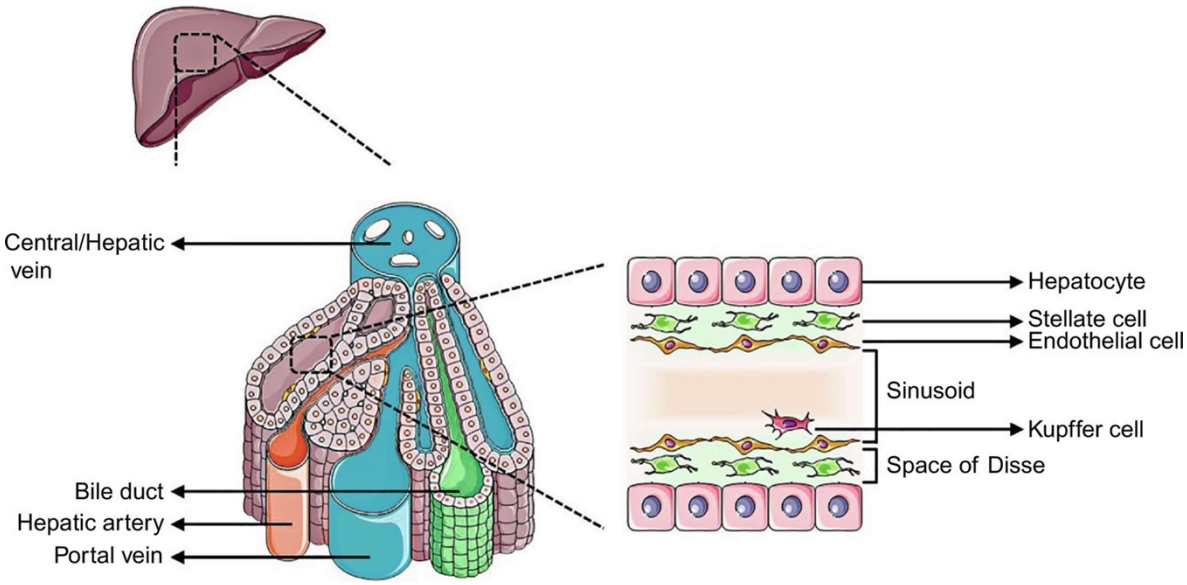

Figure 1: Hepatic architecture and different cell types that populate the liver. Reproduced with permission from Vilas-Boas, V. et al. Primary hepatocytes and their cultures for the testing of drug-induced liver injury. Adv Pharmacol 85, 1-30, doi:10.1016/bs.apha.2018.08.001 (2019).[1]

Other vessels found in the liver are bile ducts. The smallest structures of bile ducts are the bile canaliculi, which merge together to form the right and left hepatic duct, and eventually end in a common hepatic duct. The bile ducts transport bile produced by hepatocytes, which is stored in the gallbladder or drains directly into the duodenum through the hepatic duct for absorption of nutrients.

Table 1: Main cell types and their functions in the adult liver. Reproduced with permission from *Si-Tayeb, K., Lemaigre, F. P. & Duncan, S. A. Organogenesis and development of the liver. Dev Cell 18, 175-189, (2010).*[2]

Cell Type	Position in Liver	Function
Hepatocyte	Parenchyma	~70% of liver cell population Protein secretion Bile secretion Cholesterol metabolism Detoxification Urea metabolism Glucose/glycogen metabolism Acute phase response Blood clotting
Cholangiocyte/bile duct cell	Duct epithelium	~3% of liver cell population Form bile ducts to transport bile Control rate of bile flow Secrete water and bicarbonate Control pH of bile
Endothelial cell	Vasculature	Form veins, arteries, venioles, and arterioles Control blood flow Contribute toward parenchymal zonation
Liver sinusoidal endothelial cell	Sinusoids	~2.5% of lobular parenchyma Form sinusoidal plexus to facilitate blood circulation Highly specialized Allow transfer of molecules and proteins between serum and hepatocytes Scavenger of macromolecular waste Cytokine secretion Antigen presentation Blood clotting
Pit cell	Liver natural killer cells	Rare Cytotoxic activity
Kupffer cell	Sinusoids	~2% of liver Scavengers of foreign material Secrete cytokines and proteases etc.
Hepatic stellate cell	Perisinusoidal	~1.4% of liver cells Maintenance of extracellular matrix, Vitamin A, and retinoid storage Controls microvascular tone Activated to become myofibroblast Contributes toward regenerative response to injury Secretion of cytokines

1.1.2 Liver functions

Liver is a complex organ with diverse functions critical for survival. It receives blood flow from the intestines, spleen and pancreas. Thus, there is a considerable nutrient load entering the liver. Liver synthesizes a wide variety of proteins including albumin, coagulation factors, critical plasma proteins, apolipoproteins and many others. Lipids reaching the liver are transformed into lipoproteins and delivered to other tissues, while carbohydrates are stored in the form of glycogen, which is the main regulator of blood glucose levels between meals. Additionally, liver synthesizes bile for the absorption of lipophilic nutrients and fat, and it regulates, synthesizes and transports cholesterol. Furthermore, it is involved in the metabolism and excretion of hormones, exogenous compounds and metabolic waste. Finally, liver is also responsible, in part, for proper brain function as it regulates levels of glucose and ammonia in the blood, dysregulation of which could cause hepatic encephalopathy and comma. Hepatic functions are summarized in Table 1.

1.2 INBORN ERRORS OF METABOLISM

Inborn errors of metabolism (IEM) are genetic disorders that cause disruption of cellular biochemical processes. The majority of IEM affect the synthesis and/or the function of an enzyme, which result in the reduction or inhibition of conversion of substrates into products. In many cases, this disturbance causes accumulation of toxic compounds either intracellularly or systemically. The first time that IEM were reported was in 1909 by Sir Archibald Garrod in a lecture and later a book chapter entitled "Inborn errors of metabolism". In the book chapter, he predicts that "they apparently result from failure of some step or other in the series of chemical changes which constitute metabolism, and are in this respect most nearly analogous to what are known as malformations by defect".[3] The most common liver-based IEM are the urea cycle disorders (UCD), Crigler-Najjar syndrome type I, alpha-1 antitrypsin (A1AT) deficiency and phenylketonuria. For the purpose of this literature review, only UCD will be further discussed.

1.2.1 Urea cycle disorders

1.2.1.1 Background

Dietary proteins, amino acids and nitrogen-containing metabolites produced by the bacteria in the intestinal track are the main source of nitrogen in the human body. Liver is responsible for maintaining the nitrogen balance by redirecting it to be used for synthesis of proteins, pyrimidine, purines and carbohydrates. The excess nitrogen is excreted in the form of urea through the circle, which is also the only endogenous source of ornithine, arginine and citrulline. The five catalytic enzymes that comprise the urea cycle are predominantly expressed in periportal hepatocytes and are the following: carbamoyl phosphate synthetase 1 (*CPS1*), ornithine transcarbamylase (*OTC*), argininosuccinate synthetase 1 (*ASS1*), argininosuccinate lyase (*ASL*), arginase 1 (*ARG1*), with the first two catalysing the reactions in the mitochondria, and the others in the cytosol. The cycle additionally requires the presence of one cofactor-producing enzyme, called N-acetyl glutamate synthetase (*NAGS*) which is necessary to activate CPS1, and ornithine translocase (*ORNT1*), needed for the entry of ornithine into the mitochondrion and the exit of citrulline to the cytosol (Figure 2). Genetic mutations in any genes involved in the urea cycle can result in severe deficiency due to the absence of alternative pathway for metabolite clearance. OMIM entries have been registered for each deficiency (same order as mentioned above, #237300; #311250; #215700; #207900; #207800; #237310; #238970). The cumulative incidence of UCD is estimated to be 1:35,000 – 1:69,000[4,5]; nevertheless, the reality might be underestimated due to poor screening and diagnosis. Clinical presentation of the deficiency could occur at any age with acute, chronic or intermittent manifestations. Approximately half of the patients exhibit neonatal onset; cases characterized by high mortality rates ranging from 25% to 50%.[6]

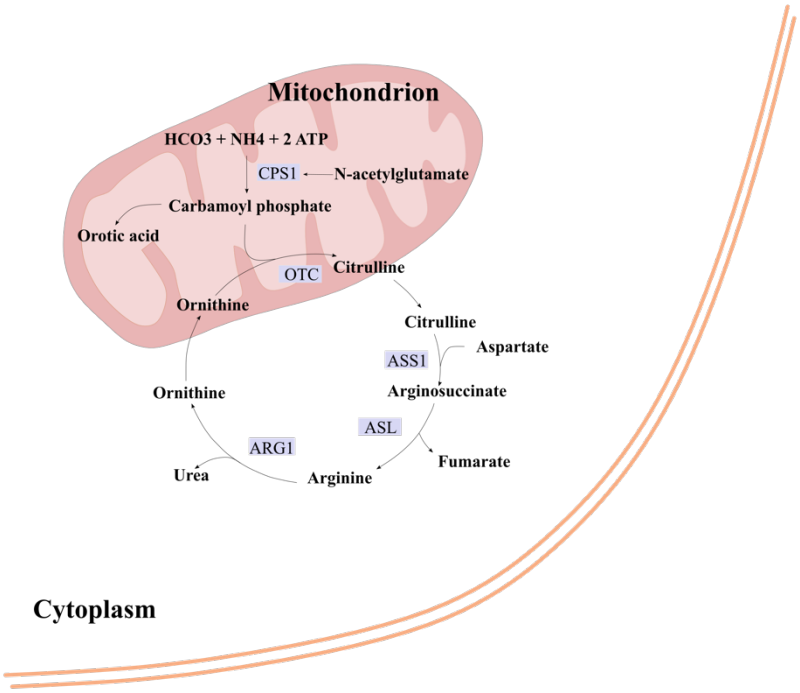

Figure 2: Urea cycle reactions showing the production of urea from ammonia. CPS1:carbamoyl phosphate synthetase 1; OTC: ornithine transcarbamylase; ASS1: arginosuccinate synthetase 1; ASL: arginosuccinate lyase; ARG1: arginase 1.

This thesis centres around OTC deficiency (OTCD) which is the most common UCD, and to a less extent around CPS1 deficiency (CPS1D). OTC is a mitochondrial enzyme that catalyses the carbamylation of ornithine leading to the formation of citrulline and inorganic phosphate in the urea cycle. OTCD is an X-linked disorder and exhibits recessive inheritance. Therefore, affected males frequently have a more severe disease than females, in whom, the second X chromosome may partially compensate for the mutated allele if not also mutated. The gene spans over 73 kb distributed into 10 exons and 9 introns. Mutations in the *OTC* gene have been identified throughout all regions. By January 2021, more than 490 mutations were listed in the Human Gene Mutation Database[7] with the majority being single-base pair substitutions causing missense or nonsense mutations (66%). A lower percentage consists of small insertion or deletions (12.5%), larger deletions (9%), splice site mutations (10.9%) as well as complex rearrangements or regulatory mutations. According to the Swedish National Board of Health and Welfare (Socialstyrelsen), the prevalence of OTCD is estimated to be one in every 40,000 – 100,000 live births which can be translated to 1-3 affected children per year in Sweden. However, there are most probably more OTCD individuals that are never diagnosed, especially those with mild symptoms.[8] The prevalence differs greatly between countries and has been reported to be 1:70,000, 1:62,000 and 1:17,000 in Finland, Italy and the USA, respectively.[5,9,10] Finally, the mortality rate of OTCD is extremely high in neonatal onset cases (74%), while significantly lower in late-onset (13%).[11]

CPS1D occurs due to mutations in the *CPS1* gene which encodes the carbamoyl phosphatase synthetase 1 enzyme. This is present in the mitochondria and catalyses the conversion of ammonia into carbamoyl phosphate. CPS1D is a rare, autosomal, recessively inherited metabolic disorder and is considered the most severe among UCD. The prevalence differs worldwide and ranges between 1:526,000-1:1,300,000.[12]

UCD-affected patients are frequently healthy at birth, and exhibit clinical manifestations a few days later. Symptomatology starts with lethargy and unwillingness to feed, and as hyperammonemia progresses, which is the most common clinical sign of UCD, vomiting, hypothermia, seizures and coma frequently appear. The spectrum of symptoms of UCD is broad, in some cases unspecific, as mirrored in Table 2. The severity and onset of these genetic defects heavily depend on the affected gene in the cycle, as well as the accountable pathogenic variant. Notably, there have been reports on partial OTCD and CPS1D, where family members with same genotype exhibited different grades of disease gravity, implying the existence of other contributory factors.[13-15] Authors in one report suggested that trans-acting elements, gene duplication or non-inherited epigenome might be among those factors, after having excluded potential contribution of other single-nucleotide polymorphisms (SNP), promoter and enhancer sequences.[15]

Manifestations of the disease might initiate at any time in life and successfully rescued patients from hyperammonemic crises are chronically at risk for repeated bouts of high ammonia levels. Finally, hyperammonemia can be triggered by catabolic events (*e.g.* infections, fever, vomiting, gastrointestinal bleeding, intense physical exercise), certain medication (*e.g.* chemotherapy, high-dose glucocorticoids), protein overload or the transition from intrauterine to neonatal life.

Table 2: Clinical symptoms of acute and chronic presentations of UCD. Modified and reproduced with permission from *Haberle, J. et al. Suggested guidelines for the diagnosis and management of urea cycle disorders: First revision. J Inherit Metab Dis 42, 1192-1230, (2019).*[6]

Acute presentation	**Chronic presentation**
Altered levels of consciousness	Confusion, lethargy, dizziness
Acute encephalopathy	Headaches, migraine-like, tremor, ataxia
Seizures	Learning disabilities, cognitive impairment
Vomiting and progressive poor appetite	Protein aversion, self-selected low-protein diet
Multiorgan failure	Abdominal pain, vomiting
Peripheral circulatory failure	Failure to thrive
Psychiatric symptoms	Hepatomegaly, elevated liver enzymes
Sepsis-like picture (neonates)	Psychiatric symptoms, hyperactivity, mood alteration, behavioural changes, aggressiveness

1.2.1.2 Diagnosis

The main laboratory marker for UCD is hyperammonemia defined as higher than 100 µmol/L in children and adults, and 150 µmol/L in newborns (but it can also reach ten times higher values).[16] Other markers of UCD are plasma levels of citrulline, arginine and ornithine, as well as urinary concentration of amino acids and orotic acid, based on which different types of disorders in the cycle are discriminated. For example, concentration of plasma citrulline distinguishes between proximal and distal urea cycle disorders, levels of arginine are elevated in ARG1 deficiency and normal in other UCD, and concentration of ornithine is elevated in

ORNT1 deficiency. Urinary orotic acid is usually elevated in OTCD, normal in CPS1D and sometimes elevated in ARG1 and ASS1 deficiencies. Detailed algorithms have been proposed for the distinction between different defects of the cycle.[6] Even though not considered a method of choice, enzyme activities can be assessed from liver (all enzymes), intestines (CPS1, OTC), red blood cells (ASL, ARG1) or fibroblasts (ASS1, ASL). Finally, mutation detection through DNA and/or RNA analysis, as well as investigation of known regulatory domains is highly recommended.[6]

In case of affected parents, prenatal screening can also be carried through mutation analysis using DNA from chorionic villous sample or amniotic fluid cells or enzyme analysis.[6] Finally, limited literature is available on the benefits of newborn screening programs. It would be expected that UCD patients would not benefit from newborn screening as severe hyperammonemia has very early onset, often before newborn screening results could be returned.[6] Currently in Sweden, ASS1, ASL and ARG1 deficiencies are UCD included in the newborn screening, along with 21 other genetic disorders.

1.2.1.3 Treatment

Short-term management: Hyperammonemia can be a life-threatening condition, and regardless of its aetiology, it should be managed as quickly as possible. The damage caused by a hyperammonemic crisis can be irreversible and it is associated with the duration[17,18] and the magnitude of ammonia rise[19-21]. Ammonia detoxification is performed with interruption of protein intake, glucose administration to prevent catabolism, and ammonia scavengers (L-arginine, sodium benzoate, sodium phenylbutyrate etc.). If hyperammonemia is not manageable with the above, extracorporeal detoxification is usually applied.[6]

Long-term management: Overall aims for long-term treatment of patients with UCD is to achieve metabolic stability, normal physical and mental development limiting of chronic complications, as well as providing a good quality of life to the patients.

Cornerstones of UCD management are reduction of dietary protein and administration of ammonia scavengers. Low protein diet is recommended as an effort to minimize the nitrogen load on the urea cycle. This recommendation mainly comes from the physiological rationale and clinical practice and experience, rather than scientific studies.[6] The level of restriction needs to be tailored depending on each patient, their physical activity and protein tolerability. Noteworthy, over-restriction might cause metabolic imbalances and growth retardation. Additionally, patients often require supplementation of essential amino acids, vitamins and minerals.[6] Drugs routinely used for the treatment of UCD are ammonia scavengers (*e.g.* sodium benzoate, sodium phenylbutyrate, sodium phenylacetate, glycerol, phenylbutyrate) that provide an alternative pathway for nitrogen disposal.

Unfortunately, in most of UCD cases the steps described above are not sufficient to keep the patient metabolically stable. The only currently established practice for longstanding restoration of urea cycle is orthotopic liver transplantation (OLT), where the defective liver is replaced with a proficient one. Even though OLT has been proven to be efficient and endowed as treatment for a broad range of liver pathologic conditions, it is limited by the shortage of available liver donors. In order to partly deal with the scarcity of available transplants, alternatives to cadaveric OLT are used, such as split, domino or living OLT

which accounts for 15% of total transplantations.[22] Nevertheless, patients in need for liver transplantation might remain two or more years on the waiting lists and the mortality rate is greater than 10%[23], a fact which highlights the urgent need for alternative therapies to OLT. Cell-based therapy is considered an alternative treatment for patients with either liver failure or metabolic hepatic diseases. The idea of hepatocyte transplantation (HTx) as a potential therapy for irreversible liver defects was introduced in 1977 when intraportal fusion of hepatocytes improved hyperbilirubinemia in UDP-glucoronosyltransferase-deficient rats.[24] Since then, HTx has emerged as an attractive option to sustain patients awaiting OLT. A number of studies reported a positive impact of HTx with improved relevant clinical parameters when the therapy was applied in patients with acute or chronic liver disease or metabolic hepatic disorders.[25] The first patient to receive transplanted allogeneic hepatocytes as a treatment for OTCD was a 5-year-old male. Ammonia and glutamine levels were normalized within 48 hours post cell infusion, and OTC activity was detectable in a biopsy taken on day 28, while it was completely null before transplantation. However, the patient died 42 days later due to bacterial pneumonia and metabolic crisis.[26] Later reports indicate that HTx provided temporary metabolic stability and relief of hyperammonemia attributable to OTCD.[27,28] HTx was used in an attempt to correct several other liver metabolic diseases or "bridge" patients to OLT (summarized in Table 3).[23]

Table 3: Clinical transplants for metabolic liver diseases. Reproduced with permission from Strom*, S. C. & Ellis, E. Cell Therapy of Liver Disease: From Hepatocytes to Stem Cells. Vol. 2 (2013).*[23]

Familial hypercholesterolemia
Crigler-Najjar syndrome
Ornithine transcarbamylase deficiency
Argininosuccinate lyase deficiency
Citrullinemia
Factor VII deficiency
Glycogen storage disease, Type 1a and 1b
Infantile Refsum disease
Progressive familial intrahepatic cholestasis
Alpha-1 antitrypsin deficiency
Carbamoyl phosphate synthetase 1 deficiency
Phenylketonuria

Several advantages and disadvantages of OLT and HTx can be identified and are summarized in Table 4. Briefly, HTx is considered a minor surgical procedure since cell infusion requires minimal incision and the placement of a catheter, most commonly into the portal vein. Therefore, complications are fewer and less severe, as well as the cost of treatment is greatly reduced. Unlike OLT, HTx is not as restricted by timing. Another advantage of HTx over OLT is that patients retain their native liver and a cell graft rejection would most likely not have lethal consequences, as the patient would be returned to pre-transplant conditions. Finally, HTx can be used as a "bridge" for the patient awaiting an available organ. Among the disadvantages of HTx is the need for multiple cell infusions and the fact that there are no reports of sustained improvement in metabolic liver disease patients past 2 years. Unfortunately, both OLT and HTx are constrained by the inadequate number of available donors. At the same time, demand for liver donors is increasing with projections estimating an escalation of around 23% in the next 15 years.[29] Discussions regarding potential sources

of hepatocytes centre around xenotransplantation, immortalized hepatocytes and stem cell-derived hepatocytes. The latter will be further discussed in the next section.

Table 4: Advantages and disadvantages of orthotopic liver transplantation (OLT) and hepatocyte transplantation (HTx).

OLT	**HTx**
Unquestionable success	Less invasive
Highly invasive	Reduced cost
Extensive recovery period	Less severe complications
More severe complications	Patient retains native liver
Timing is critical	Potential rejection is not necessarily lethal
Shortage of available organ donors	Can be used as a "bridge" for OLT
	One organ can be used for multiple patients
	Timing is not so critical
	Multiple infusions are required
	No data regarding the long-term efficacy

1.3 STEM CELLS AS A POTENTIAL SOURCE OF HEPATOCYTE-LIKE CELLS

A critical shortage of available liver or hepatocyte donor for treating liver diseases signifies the demanding need for the identification of potential graft sources. Hepatocyte-like cells (HLC) generated from various types of stem cells might supply with a promising solution, as described in this section. Their additional utility in disease modelling is explored in the next parts of this thesis.

It is not clear when and by whom the term "stem cells" was first used, however the main properties of stem cells were defined in the 1960s by Ernest McCulloch and James Till, who worked with hematopoietic stem cells. Key characteristics of a cell to be defined as stem cell are the abilities of self-renewal and differentiation into mature cells.[30,31] Milestones in the stem cells research are studies conducted by Sir John Gurdon in the early 1960 who demonstrated that the cells are not committed to a differentiated status, but can be turned back to an earlier stage of the developmental process. Such principle was proven by the injection of a nucleus of a differentiated cell into an egg cell from which the nucleus has been previously removed. The modified egg could differentiate into all cell types of the organism and generate a fertile adult frog.[32]

Blastocyst is a structure formed during the early development of mammals. It consists of around 300 cells forming two main compartments, the inner cells mass (ICM) and the trophoblast. The first consists of embryonic stem cells (ESC) and gives rise to all cells of the embryo during the development, but not the extra-embryonic tissues such as the placenta, while the latter develops into perinatal layers. The ability of ESC to differentiate into all three germ lineages (endoderm, mesoderm and ectoderm) was discovered and described as early as in the 1960s and 1970s.[33,34] Later, this was further investigated by Evans and Kaufman who first isolated and cultured ESC from mouse blastocysts in 1981.[35] These advancements opened the door to the generation of mouse models of various genetic backgrounds. Genes of interest could be modified in ESC, and consequently injected into a blastocyst to develop into an adult mutant mouse producing mouse models that recapitulate various genetic pathologies. However, the immaturity of genome editing techniques at that time made the aforementioned a cumbersome procedure. Several years later, human ESC were isolated and cultured by Thomson and colleagues[36]. This work sparked enthusiasm because for the first time, researchers could theoretically generate any cell population of the human body in unlimited amounts, given the fact that ESC have the abilities to differentiated into all three germ layers and indefinitely self-renew. Additionally, the use of human ESC in research could provide tools for better understanding of developmental biology, disease mechanism and drug discovery.

Later, Yamanaka and associates used somatic cells to generate induced pluripotent stem cells (iPSC) through the forced expression of four transcription factors.[37,38] Discoveries that helped to mitigate the controversial ethical concerns that ESC pose. Additionally, iPSCs are considered advantageous over ESC because patient's own cells can be used, meaning that personalized cellular therapy would be possible, theoretically circumventing the need for immunosuppressive treatment. Different cell sources have been used to produce iPSC, such as skin fibroblasts[38], blood cells[39], adipose-derived stem cells[40], hepatocytes[41], pancreatic β-cells[42], keratinocytes[43] and others. The choice of somatic cell source is affected by factors, such as invasiveness to obtain the cells and epigenetic memory, as it has been shown that

somatic cells may retain their epigenetic memory and have enhanced differentiation potential into the cell type of origin.[42,44]

Understanding the processes during natural human embryogenesis contributed to protocols for hepatic differentiation of pluripotent stem cells.[45] As mentioned above, embryonic stem cells have the ability to differentiate into all three germ layers after gastrulation. The liver, lung, pancreas, gastrointestinal track and thyroid are developed from the definitive endoderm lineage. In embryonic development, definitive endoderm initially forms as an epithelial sheet of cells at the ventral surface of the embryo. Later, this sheet of cells unfolds creating the foregut and the hindgut leading to gut tube formation. Different parts of the gut tube, anterior-posterior and dorsal-ventral axes, give raise to different tissues. The liver develops from the prospective ventral endoderm part of the foregut.

The first *ex vivo* hepatic differentiation protocols relied on embryoid body formation and spontaneous differentiation with the use of particular growth factors.[46-48] However, this process lacks reproducibility and is characterized by generation of mixed populations of differentiated, partially differentiated and undifferentiated cells. Later, protocols involved more targeted differentiation approaches. The majority of the current protocols follow a 3-step procedure; definitive endoderm induction, mainly using ActA, FGF, BMP4, WNT3A, followed by hepatic specification into hepatoblasts, primarily by exposure to FGF, BMP4 and HGF, and finally differentiation into hepatocyte-like cells mainly with HGF, oncostatin M and dexamethasone (Figure 3).[49] Conditions vary involving 2D culture or 3D organoids, as well as growing in suspension or combinations of the above.[50-52] Stem cell-derived HLC have been used in a variety of applications, including disease modelling, drug metabolism, non-coding RNA, as well as in studies related to infectious and hepatic diseases.

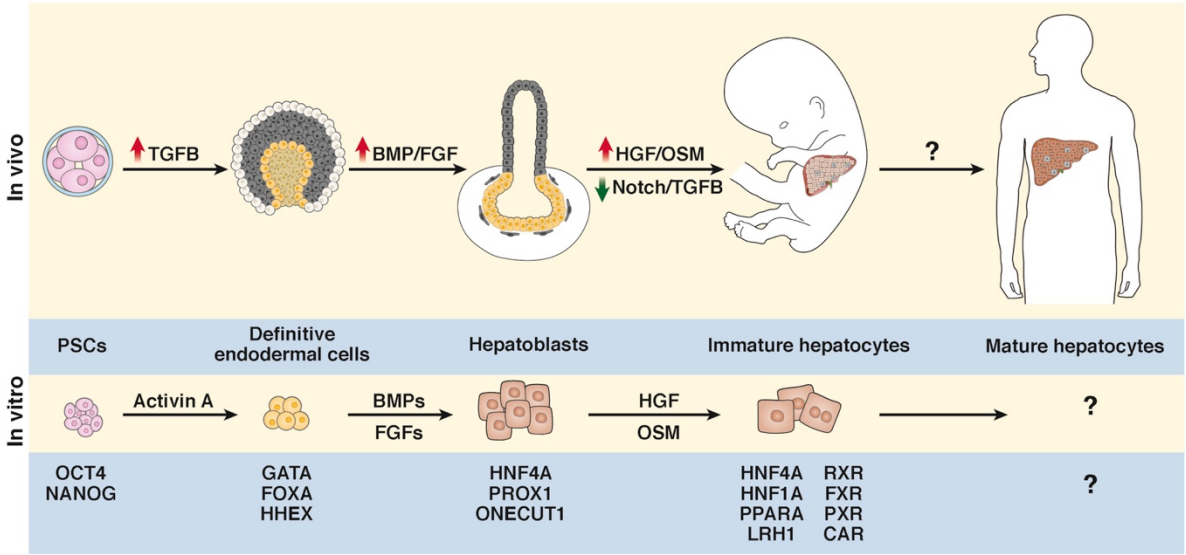

Figure 3: Established factors regulating each phase of hepatic differentiation. Question marks indicate pathways under investigation. Reproduced with permission from Chen C, Soto-Gutierrez A, Baptista PM, Spee B. Biotechnology Challenges to In Vitro Maturation of Hepatic Stem Cells. Gastroenterology. 2018 Apr;154(5):1258-1272.[49]

However, despite the great potentials that stem cells might offer, there are considerable roadblocks. Stem cell-derived HLC lack maturity as they resemble more functions of fetal hepatocytes, rather than those of the adult counterparts.[53,54] Specifically, they often maintain residual expression of alpha-fetoprotein (*AFP)* and *CYP3A7* which are considered fetal

markers, and fail to reach mature levels of essential genes, such as Albumin and CYP450 genes, critical for the metabolism of endogenous and exogenous compounds. The limited capacity of hepatic differentiation would be expected if we consider that *in vitro* differentiation protocols are oversimplified procedures involving limited number of growth factors, cytokines and other molecules usually lasting around a month. On the contrary, natural hepatogenesis takes nine months of prenatal development, and as long as two years after birth for liver maturation receiving countless signals and being exposed to numerous growth factors, molecules and others, as well as dynamic changes in the body (nutrients, circulation, microbiota etc.). Research efforts focus on several aspects to improve differentiation and maturation of lab-made hepatocytes, such as growth and transcription factors, microRNA, small molecules, and microenvironment (extracellular matrix, co-culture and dynamic culture), as illustrated in Figure 4.[49] Additionally, high throughput screenings have been used to elucidate mechanisms and identify small molecules to enhance HLC maturation either alone[55,56] or in combination with CRISPR (clustered regularly interspaced short palindromic repeats) libraries.[57] Finally, HLC maturation might likely benefit and be enhanced from transplantation of cells *in vivo*. An example of such study attempted to transdifferentiate fibroblasts into HLC, overcoming the pluripotent state, by transplanting the cells into the liver of FRG mice (mouse model of liver humanization described in a next section - FRGN – A liver-humanized mouse model). Beside the discouraging results of being able to repopulate only 2% of the mouse liver after 9 months of monitoring, investigators showed enhancement of cell maturity after *in vivo* incubation, suggesting a potential way to improve maturation of HLC.[58]

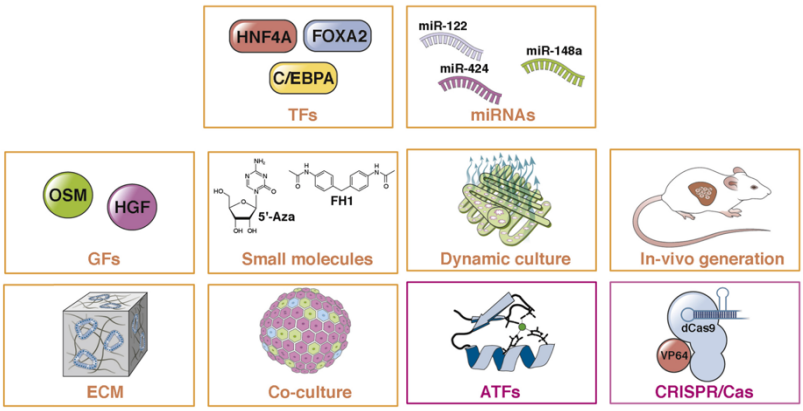

Figure 4: Potential tools to enhance differentiation and maturation of generated hepatocyte-like cells. TF: transcription factors; miRNA: microRNA; GR: growth factor; ECM: extracellular matrix; ATF: activating transcription factor; CRISPR: clustered regularly interspaced short palindromic repeats; Cas: CRISPR-associated protein. Modified and reproduced with permission from Chen C, Soto-Gutierrez A, Baptista PM, Spee B. Biotechnology Challenges to In Vitro Maturation of Hepatic Stem Cells. Gastroenterology. 2018 Apr;154(5):1258-1272.[49]

Defining hepatic maturation: In order to generate HLC which resemble adult mature hepatocytes, features of hepatic maturity need to be defined. First and foremost, adult primary hepatocytes are required to be used for comparison reasons. Additionally, freshly isolated cells are preferable because hepatocytes are vulnerable to cryopreservation effects (viability, plating efficiency and hepatic functions).[59] Furthermore, there is considerable variability between individuals, and consequently standards of maturity should be set based on primary

cells from multiple donors; yet, it is generally agreed that certain genes are expressed during the prenatal period, and decreased later in the postnatal, or the opposite; functions might be at negligible levels during fetal development, and note significant increase after birth or in the first years of life.

First characteristic of mature hepatocytes is their morphology with epithelial features (polygonal with microvilli on cellular membrane), as well as polarization and polyploidization. Furthermore, relevant HLC for clinical and disease modelling would require metabolic and secretory functions at similar levels to adult primary hepatocytes. Drug-metabolizing enzymes are essential with CYP3A4 being the most abundant in adult liver, while in general those belonging to families 1, 2 and 3 are accountable for 70-80% of phase 1 metabolism.[49] These can be evaluated based on gene expression levels or preferably tested *in vitro*.[60] Other *in vitro* assessments that could serve as indicators of hepatic maturity are bile acid synthesis, glycogen storage, urea cycle function, as well as serum protein synthesis such as albumin, A1AT, fibronectin, transferrin, coagulation factors etc. Moreover, criteria of differentiation efficiency are also considered cholesterol metabolism and lipid uptake. Last but not least, successful differentiation requires the repression of pluripotency markers which can be assessed with various means, most commonly gene or/and protein expression levels. Finally, if cells are intended for transplantation *in vivo*, characteristics such as engraftment and repopulation capacity, restoration of liver function and tumorigenicity are of importance.

Among other hurdles that HLC face is the tumorigenic potential that may not be eliminated completely. Even a few undifferentiated cells can result in the formation of a teratoma. This could potentially be removed with more efficient and effective differentiation protocols so that the generated hepatocytes satisfy clinical and research requirements and standards. Finally, aberrant or incomplete reprogramming of somatic cells might contribute to the impaired differentiation into the cell type of interest.

1.4 EXPERIMENTAL MODELS

Experimental models are essential in biomedical and biotechnological research in order to understand the development, progression and causation of diseases, as well as to perform drug testing and develop general medical procedures. In order to test hypotheses, two broad categories of experimental models are used: *in vitro* and *in vivo*.

In vitro (Latin: *in glass*): Refers to procedures performed outside of a living organism in a controlled manner. These procedures might include microorganisms, cells, or biological molecules that have been isolated from their biological surroundings. Advantages of *in vitro* experiments are their relative simplicity, species specificity (*e.g.* the use of human cells), reduction of animal use and the convenience of utilizing in high-throughput screening methods. However, in many instances there is a lack of appropriate platforms. Additionally, in many cases it might be challenging to translate *in vitro*-derived experimental results to whole organisms; therefore, before applying to humans, safety and efficacy of treatments must be tested in a series of *in vivo* studies.

In vivo (Latin: *within the living*): Refers to experimental procedures using whole organism, including animal studies and clinical trials. *In vivo* experiments are often employed over *in vitro* because the effect can be assessed overall in a whole living subject. However, there are significant differences between the commonly used laboratory animal models and humans, related to metabolism, excretion, repair pathways, genetic sequences and others. Therefore, results using animal models might in some instances not faithfully predict how they will be translated to humans. Perhaps one of the most notable examples of poor translation is Fialuridine, a compound administered in a clinical trial for hepatitis B virus (HBV). The drug showed little to no toxicity in preclinical animal studies, but the administration to humans caused hepatic failure, neuropathy, myopathy and pancreatitis. Despite immediate discontinuation of the medication, five out of seven patients died, while two others survived after emergency liver transplantation.[61] Recent advances have allowed the generation of "humanized-animals" which might be more appropriate in many settings for preclinical studies. Those are described in the next chapter with focus on liver-humanized mice.

1.4.1 *In vitro* models

In vitro models have led to significant insights into pathogenesis and treatment of liver diseases. Some have been traditionally used for decades (*e.g.* immortalised cell lines, primary cells) while others are emerging as promising avenues (*e.g.* 3D models, stem cell-derived HLC, organ-on-a-chip). Advantages, disadvantages and applications of these platforms are briefly discussed below.

Primary hepatocytes: Primary hepatocytes are widely used in hepatological studies. Initially, they were isolated with mechanical forces using glass homogenizers with loose pestles[62], glass beads in a Kahn shaker[63] or forcing the liver through a cheesecloth[64], which were proven to damage cellular integrity of isolated cells and cause loss of function. Later, Berry and Friend demonstrated that perfusion of rat livers through their existing vascular system with collagenase and hyaluronidase could increase cell yield, as well as maintain structural integrity. The procedure was further improved when Seglen introduced a two-step perfusion

procedure; the first with Ca^{2+}-free buffer which allowed the disruption of desmosomes that hold the cells together, while the second step with collagenase and addition of Ca^{2+}, required for optimum collagenase activity[65,66]. Strom and others modified the existing protocols for rat hepatocyte isolation into applicable procedures for human liver[67-70], which are still widely used.

Conventional monolayer cultures (2D) of primary hepatocytes are considered the standard method for toxicity and drug metabolism studies because they resemble morphologically, functionally and biochemically *in vivo* physiology to a satisfactory degree.[60,71] Yet, they are characterized by several shortcomings with the most important being the abolishment of cell-cell and cell-extracellular matrix interactions, during and after isolation, which triggers the cell de-differentiation.[1] De-differentiation is signalled by loss of morphology, cell polarity and hepatic functions. Given the immense demand for *in vitro* platforms, efforts to decrease de-differentiation have been made through 3D-organoid formation alone[72], or in combination with co-culture of non-parenchymal cells[73]. Another strategy to decrease de-differentiation is the culture of hepatocytes in-between two layers of extracellular matrix, mainly collagen, as a way to regain cell polarity.[74] Furthermore, bioreactor-based models, such as stirred tank bioreactors, are also used for aggregation of primary hepatocytes into spheroids, which better sustain the differentiated phenotype, polarity and metabolic performance.[75] Among other drawbacks of primary hepatocytes are their inability to proliferate and expand, early senescence, as well as vulnerability to cryopreservation.[76,77] Finally, the shortage of available primary hepatocytes, in particular cells from specific genetic disorders, such as UCD studied in this thesis, indicates the need for identification of other *in vitro* models.

Cancer cell lines: Liver tumour cell lines are acquired from liver cancers, particularly from hepatocellular carcinoma. They are largely employed in research studies because of their simplicity, reproducibility and proliferation capacity providing theoretically an infinite cell supply. However, they display restricted genotypic variability because they are procured from a single individual, and they do not perform hepatic functions at comparable levels to primary hepatocytes.[78] Furthermore, cancer cells have dysfunctional apoptotic pathways due to their tumorigenic origin, and have been reported to maintain the genomic and transcriptomic landscape of primary human cancers.[79]

Immortalized human hepatocyte lines: Immortalized hepatocyte lines are commonly derived from healthy primary hepatocytes. They can proliferate and be cultured for a prolonged period of time because they have evaded normal cellular senescence through immortalization strategies. Among the most common hepatocyte immortalization strategies are the overexpression of viral oncogenes or forced expression of human telomerase reverse transcriptase (hTERT), or a combination of both. Several fetal, neonatal and adult hepatocyte lines have been established. They generally display reduced or limited hepatic functions (*e.g.* loss of CYP450 potential), genome alterations, abnormal proteome and loss of morphological features (*e.g.* cellular polarity).[80] Still, immortalized hepatocyte cell lines have been used for various application, including CYP induction experiments, as well as studies related to HBV/HCB infection, replication and drug screening.[81] To the best of my knowledge, these platforms have not been applied for studies associated with UCD.

Organ-on-a chip: Organ-on-a-chip devices have been also proposed as *in vitro* models. They usually utilize a multichannel 3D microfluidic cell culture chip attempting to replicate *in vivo* microenvironment and homeostasis. The majority of such systems use primary hepatocytes, and to a less extent liver cancer cell lines, stem cell-derived HLC or co-culture of primary hepatocyte with non-parenchymal cells. Organ-on-a-chip models have been used in several studies and are commercially available (Zyoxel and Microliver chip from HμRel©), yet not fully validated.[80]

Precision-cut liver slices: Another alternative for *in vitro* modelling of liver diseases are precision-cut liver slices. Even though they preserve to some extent the liver structure and therefore cell-cell and cell-extracellular matrix interaction, their use is impeded by their short-term viability, loss of functions and the requirements of advanced technical skills.[82] Conclusively, the model is not widely adopted.

Stem cell-derived HLC: HLC from stem cell sources is another option for *in vitro* disease modelling that has been embraced with enthusiasm. Historically, stem cells derived from the ICM have been used for the generation of HLC. However, after the breakthrough of somatic cell reprogramming, iPSC have become more common substrate for hepatic differentiation.[83] One of the main reasons for iPSC rapidly gaining ground is that they can be derived from individuals with various genotypes, including patients with rare liver genetic disorders. Another appealing characteristic of iPSC is the relative ease with which they can be genetically engineered to obtain the desired genetic background; therefore, they can be a source of diseases-specific hepatocytes. Furthermore, iPSC can generate an indefinite cell supply that can maintain stable genomic, transcriptional and epigenetic profiles when carefully handled.[84] Even so, challenges that need to be tackled are the heterogeneity of differentiated cell populations, as well as their incomplete hepatic differentiation (discussed in a previous section - Stem cells as a potential source of hepatocyte-like cells). Even though HLC do not display the full repertoire of hepatic functions, it is recognized that they outperform many other *in vitro* models, and the attained maturation level might be sufficient for some applications.[85] A substantial number of studies have used patient-specific iPSC to model hepatic genetic disorders, including A1AT deficiency[86-89], ARG1 deficiency[90], ASS1 deficiency[91], citrin deficiency[92], Crigler Najjar syndrome[86,93], familial hypercholesterolemia[86,94-96], glycogen storage diseases[86], haemophilia A and B[97,98], Pompe disease[99], primary hyperoxaluria type 1[100], tyrosinemia type 1[86,93] and Wilson's disease[101,102]. Noteworthy, iPSC-derived HLC have also been used for high throughput screenings; platforms that enable the assessment of drug toxicity and evaluation of novel drug activities. For instance, Choi et al. generated a model of A1AT deficiency which accumulates protein aggregates due to protein misfolding. Genetically defected HLC were treated with a drug library consisting of more than 3,000 compounds, and determined through immunofluorescence microscopy, which molecules could reverse the phenotype. Among the drugs identified was Carbamazepine that had already proven to ameliorate A1AT intracellular accumulation in non-human primates, proving the robustness of such platforms.[87] In the same fashion, iPSC-HLC from a patient with familiar hypercholesterolemia were utilized to screen libraries of small molecules which could potentially reduce apolipoprotein B (apoB) production.[96] Furthermore, iPSC-derived HLC-based high throughput screenings are not limited to drug toxicity and identification of novel

treatments. They have been also used to investigate non-coding SNPs from GWAS that might cause lipid metabolic defects.[103]
Finally, in the last years there has been a burst in studies developing or utilizing organoids. These are 3D multicellular structures that can recapitulate morphological and functional features of *in vivo* physiology more faithfully than conventional 2D cultures.[50,52,104] *ASS1* deficiency, a urea cycle defect, has been modeled with patient specific iPSC-derived HLC organoids in which ammonia was elevated when compared to organoids from healthy donors. Furthermore, the phenotype was reversed with overexpression of *ASS1* indicating the potentials of disease modeling and drug screening in a fast and efficient manner.[105]

1.4.2 Animal disease models

Animal disease models are non-human animals bearing pathologies that share clinical manifestations with a specific disorder in humans. They are used for research purposes to elucidate causes, pathology, prevention or treatment of a specific disorder. Rodents (mice and rats) are the most frequently employed to investigate liver diseases, however large animals (rabbits, dogs, chimpanzees etc.) are occasionally used. Reasons for these preferences include easier maintenance and breeding due to their small size and short gestational period, relatively simple genetic engineering and shared similarities with humans. In this section, the focus will be placed on rodents only.

1.4.2.1 Animal disease models of UCD

Despite the undeniable significance of animal disease models, only a limited number of such platforms for UCD have been described. This might be justified by the high neonatal lethality of these models (with some being lethal within one day after birth[106]), as well as the difficulty to rescue them with biochemical interventions. Some models of UCD are discussed below.

N-acetylglutamate synthetase (NAGS) deficiency (OMIM 237310) is characterized by partial or complete lack of the enzyme N-acetylglutamate synthetase which catalyses the formation of the essential allosteric activator of CPS1, N-acetylglutamate, from glutamate and acetyl-CoA.[107] Two knock-out mouse models of NAGS deficiency have been described. In one study, knockout homozygous ($Nags^{-/-}$) pups could be rescued when treated with intraperitoneal injection or water supplemented with N-carbamoyl-glutamate and L-citrulline after weaning, noting 85% survival rate and allowing normal development, apparent normal development, health and reproduction. Discontinuation of the medication regime was associated with the appearance of disease symptoms, including increased ammonia, glutamine and glutamate, along with decreased citrulline, arginine and ornithine, leading ultimately to expiration within 48 hours. Conclusively, the mouse model recapitulated a severe proximal UCD and the phenotype was reversed upon biochemical intervention.[108] The other study describing *Nags* deficient mice showed that administration of N-carbamoyl-glutamate to pregnant females allowed the survival of homozygous pups for 60-120 hours, while the untreated controls perished within 24 hours. Even though both groups survived for

very short period, this study perhaps indicates that biochemical intervention might increase the window for the investigation of other potential therapeutics for NAGS deficiency.

Carbamoyl phosphate synthetase 1 (CPS1) deficiency (OMIM 237300) arises from genetic defects in the *CPS1* gene. The protein is highly abundant in the liver and converts ammonia into carbamoyl phosphate.[107] A knockout mouse model generated for this disorder faithfully recapitulated the clinical features of the disease in humans, including severe hyperammonemia within 6 hours of birth. However, the severe phenotype of the deficiency allowed the survival of *Cps1*$^{-/-}$ pups no more than 36 hours. Investigators support that "this animal is a good model of human CPS1D"[106], however the high mortality might pose hurdles for treating and even maintaining the animals. The model has not been submitted to a repository, and most probably no longer exists.[109] A later study generated a conditional disruption of *Cps1* gene which allowed the maintenance of normal animals until the gene was deleted using Cre recombinase technology. About 14 days later, affected animals developed manifestations consistent with those in CPS1D patients and survived up to 4 additional weeks.[110] Perhaps the conditional disruption of the gene might be a more appropriate approach because it leaves room for action and increases the convenience of maintaining the animals.

Ornithine transcarbamylase (OTC) deficiency (OMIM 311250) is the most common UCD and results from pathogenic variants in the *OTC* gene, responsible for the conversion of phosphate and ornithine into citrulline. Contrary to other UCD, OTCD is inherited in a X-linked manner; thus males exhibit more severe symptoms, while heterozygous females can have a quite variable spectrum of disease severity.[107] Mainly two knockout mouse models of OTCD have been used to investigate disease pathogenesis and novel therapeutics. The first, sparse-fur (*spf*) was first described by De Mars et al. and was generated spontaneously in the progeny of an irradiated male.[111] The deficiency is cause by a C>A missense variant in exon 4 leading to an amino acid substitution (H117N) that results in 10% of normal liver enzyme activity.[112] The second model, called *spf*^{ash}, shares resemblance with the *spf* mouse and displays abnormal skin and hair (*ash*).[113] The deficiency arose from G>A missense mutation in exon 4 which led to substitution of arginine into histidine (R129H) decreasing ultimately the enzyme activity to 5% of normal range due to abnormal splicing.[114] Both models display symptoms of the disease, including high plasma ammonia and glutamine, low plasma arginine and citrulline, as well as elevated urinary orotic acid permitting the study of various aspects of the disease. A new model of OTCD has been relatively recently reported (*spf-j*).[115] It was created by a spontaneous mutation in exon 3 of the *Otc* gene (c.240T>A, p.K80N). The high residual amounts enzyme activity in the mouse maintained ammonia and orotic acid at normal levels, while the profiles of glutamine, citrulline and arginine were consistent with mild OTCD. This might be a more appropriate model to study late onset cases of the disorder.

Argininosuccinate synthetase 1 (ASS1) deficiency (OMIM 215700) is caused by defects in *ASS1* gene that catalyses the condensation of aspartic acid and citrulline to form argininosuccinate.[107] Disruption of first exons in the *ASS1* coding region led to generation of a null mutant mouse of this specific genetic disorder.[116] With the complete abolishment of enzyme activity, homozygous mutants did not gain weight and expired within 24 hours after

birth. Their biochemical profiles showed 16-fold increase in plasma ammonia, as well as citrulline elevation and arginine decrease. Due to the clinical and biochemical consistency of the phenotype in the mouse model with affected humans, it has have been used in preclinical studies for the development of gene therapy treatments.[117-119] Furthermore, two independent alleles described in patients with citrullinemia have been introduced in mice to generate models of ASS1 deficiency. On the contrary to the *Ass1* knockout mouse described above, these mice displayed a milder phenotype. Some animals survived for one week after birth, while others for a longer period of time, recapitulating features of the disease (retardation in postnatal development, alopecia, lethargy, citrullinemia, hyperammonemia).[120]

Argininosuccinate lyase (ASL) deficiency (OMIM 207900) results from mutations in the gene responsible for cleavage of argininosuccinate into fumarate and arginine. Reid Sutton et. al.[121] generated a mutant mouse for ASL deficiency by creating a frameshift in mRNA. All homozygotes expired within 48 hours after birth and they displayed several biochemical hallmarks of the disease, including increased plasma ammonia, argininosuccinate, glutamine and citrulline in addition to low plasma arginine. Another mouse model of this particular UCD was created with the introduction of neomycin cassette in intron 9 of *Asl* gene. Molecular analysis demonstrated the hypomorphic nature of the genetic manipulation, as mice maintained 25% residual RNA, 25% residual protein and 16% residual enzyme activity. Nevertheless, phenotypic characterization indicated some metabolic derangements and mice survived for several days.[122]

Arginase I (ARGI) deficiency (OMIM 207800) occurs in cases of genetic defects in the gene encoding ARGI, the final enzyme of urea cycle.[107] A model of this genetic disorder has also been constructed with complete abolishment of the enzyme. Animals exhibited a severe phenotype and could be maintained alive for only 10 to 14 days postnatally. Characteristic symptoms included elevated plasma ammonia and arginine, as well as highly decreased ornithine. Additionally, histological analysis of the liver showed hepatic abnormalities, such as cell swelling and inclusions.[123] While a strong disease phenotype of the UCD was achieved, the mice were not amenable to further studies as they perished before weaning. Once again, similar to the CPS1D mouse model described above[110], researchers generated conditional knockout mice of ARG1 deficiency exhibiting some pathologic manifestations.[124]

To recapitulate, a few models of UCD currently exist. Some of those are characterized by high fatality due to their severe deficiency phenotype, complicating animal maintenance and making them less amenable to further studies, while others display a mild disease phenotype which does not cover the whole repertoire of the disease symptomatology. Yet, even if the phenotype is recapitulated to a satisfactory degree and the animals survive to an acceptable period of time, interspecies differences might cofound the interpretation of the results when translated to humans. Perhaps, humanized mouse disease models might be more appropriate in many settings for preclinical studies and they are discussed in the next section.

1.4.2.2 FRGN – A liver-humanized mouse model

The undeniable significance of disease animal models has been elaborated above. However, many aspects, including infectivity by various pathogens, differences in physiology, metabolism, cell replication and DNA repair pathways may not be replicated in animals. Creating models that would more faithfully recapitulate human diseases and physiology might contribute to the avoidance of poor translation of results from laboratory animals to humans, as unhappily has occurred in the instances of chronic hepatitis B[61] and OTCD gene therapy[125] clinical trials.

Humanized chimeras are animals that bear human elements, such as DNA, tissues, cell populations or a whole organ, useful for the study of various aspects of human diseases and the assessment of therapeutic interventions. The first example of chimeras were mice with "humanized" tumours allowing a more accurate understanding of human tumour biology.[126]

A common feature of humanized mice is the need for a profoundly immunodeficient background. Such models are severe combined immunodeficient (SCID) strains that lack T-, B- and NK cells allowing the xenograft of human cells.[127] Later, knockout of the common γ-chain of the interleukin 2 receptor (*Il2rg*) made the generation of mice nearly incapable of rejecting human cells.[128] Another common characteristic of a mouse model for liver humanization is a genetic modification that allows the destruction of the native murine hepatocytes, thereby providing a regenerative environment that favours the replacement of murine hepatocytes with human cells. The first mouse model generated for this purpose was albumin-promoted, urokinase plasminogen activator (uPA) mouse.[129] The expression of uPA activates plasminogen which subsequently activates plasmin and induces intracellular proteolytic damage in hepatocytes, resulting in hepatic injury.

Later, another mouse model was generated based on fumarylacetoacetate hydrolase (*Fah*) deficiency with a severely immunodeficient background (*Fah*$^{-/-}$/*Rag2*$^{-/-}$/*Il2rg* $^{-/-}$, FRG mouse).[130] Disruption of *Fah*, a gene involved in tyrosine catabolism, results in the accumulation of toxic compounds. Although quite toxic, these compounds have what is called a limited bystander effect, since they have short half-lives and do not exit the cells where they are produced. Therefore, FAH-proficient cells are not affected by the toxic compounds within FAH-deficient cells, even though they may be immediately adjacent. This is also demonstrated by the observation that non-parenchymal cells, such as biliary, endothelial and Kupffer cells are quite normal in the mouse liver.[131] Accumulation of the aforementioned toxic compounds can be prevented with the administration of the protective drug 2-(2-nitro-4-trifluoromethylbenzoyl)-1, 3-cyclohexanedione (NTBC)[132] which blocks tyrosine metabolism upstream of *Fah* (Figure 5). When the drug is withdrawn after transplantation of donor FAH proficient hepatocytes, there is a selective replication pressure that allows the donor cells to proliferate and repopulate the liver over time. Later modifications of the FRG mouse strain included crossing with the non-obese diabetic (NOD) background generating the FRGN mouse which was proven to have higher acceptance of human cells.[133] Other transgenic mouse models for liver-humanization are the TK-NOG[134] and AFC8 models[135], both rely on the conditional activation of hepatotoxic transgenes, ganciclovir and AP20187, respectively.

A standard method for generating liver-humanized FRGN mice is well-established, and has been applied in several parts of the research work presented in this thesis. The animals are "healthy" while on NTBC and the drug is withdrawn after transplantation. One to three days

prior to transplantation mice are injected with a uPA-expressing adenoviral vector, which temporarily damages the liver and importantly, disrupts the endothelial cells leading to improved initial cell engraftment. Normally, 0.25-1x10^6 cells are transplanted into the spleen of 6-8 weeks old mice. The relative level of repopulation is estimated based on the amount of human albumin in the mouse blood (each 1 mg/ml human albumin corresponds to approximately 20% liver repopulation).[133] Noteworthy, among the shortcomings of this liver chimeric mouse is that it requires significant experience with hepatocyte transplantation and animal handling. Additionally, replacement capacity greatly depends on the donor cells, however the majority of donor cases can, if not fully, repopulate the liver to some extent. In the event of incomplete liver humanization, cautious study design, performance and result interpretation are prerequisites as residual murine hepatocytes might considerably interfere with the read-out. Similarly, host extra-hepatic tissues, such as kidneys or intestines, might confound the results.

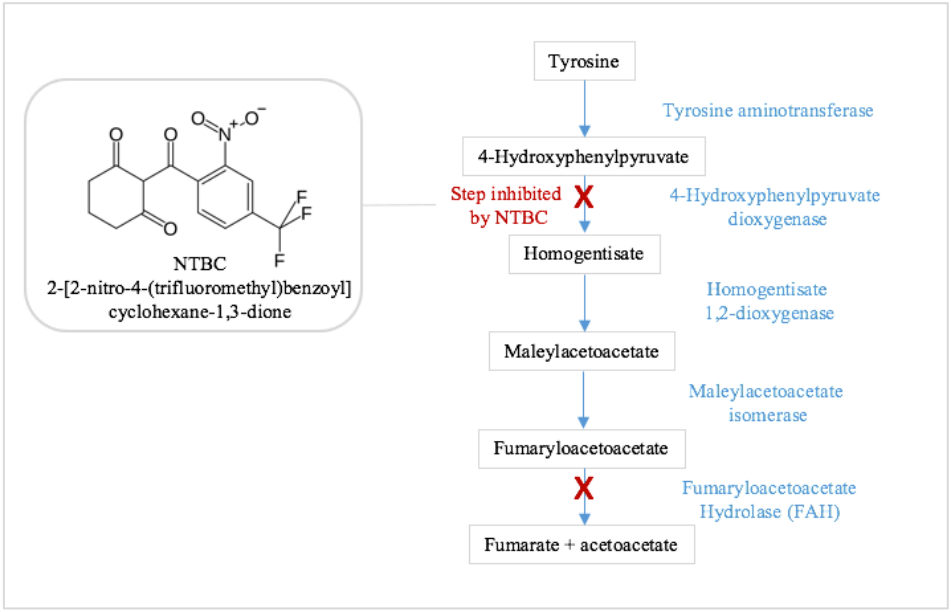

Figure 5: Enzymatic reactions of tyrosine metabolism and NTBC site of action. NTBC inhibits 4-hydroxyphenylpyruvate dioxygenase (HPPD) preventing the production of maleylacetoacetate and fumaryloacetoacetate, which have the potential to be converted into other toxic compounds.

When studies are carefully designed and data meticulously interpreted, liver-humanized mouse models represent a useful platform for diverse applications. Noteworthy, they can be humanized not only with genetically "normal" hepatocytes, but also with hepatocytes from individuals with genetic defects. For instance, researchers established a model of paediatric liver cancer by transplantation of patient-derived tumour xenograft into FRG mice. The chimeric animals recapitulated the histologic, genetic, and biological features, including the metastatic behaviour of primary tumours pointing out a direction for the exploration of new therapies.[136] The establishment of model diseases through such strategies enables the research in the gene therapy and gene editing fields. Liver humanized mice were used in an early proof-of-concept study of *in vivo* CRISPR-mediated genome engineering targeting proprotein convertase subtilisin/kexin type 9 (*PCSK9*).[137] Circulating human PCSK9 protein was greatly reduced after nearly 50% on-target mutagenesis. Authors concluded that the work provides important information regarding efficacy and safety of CRISPR-mediated therapy. Perhaps the statement regarding safety might be rather superficial, since the study investigated potential undesired mutagenesis at only the 10 top *in silico* predicted sites.

Another interesting study repopulated FRG mice with hepatocytes from an OTCD affected patient.[138] Following adeno-associated virus (AAV)-mediated delivery of the required editing reagents, cleavage rates of targeted *locus* reached 72%, while homology-directed repair (HDR)-based editing up to 29%. Finally, the use of liver-humanized models for gene therapy and editing might elucidate differences in DNA repair machinery and transgene integration.[139]

An additional example that further demonstrates the utility of humanized models is a study where different AAV capsid pseudotypes and their transduction profiles were investigated.[140] Interestingly, vectors effective in transducing murine hepatocytes *in vivo*, showed poor (20 times lower) effectiveness in human cells in liver-humanized mice indicating interspecies preferences. Moreover, inconsistencies between viral transduction efficiency between *in vitro* and *in vivo* experiments were reported. Taken all together, the use of a humanized mouse model in this instance assisted in overcoming interspecies differences and in emulating the native setting of hepatocytes, as well as providing more physiological functions and gene expression profile than in *in vitro* settings.

The use of humanized mouse models has been additionally proposed as way to improve hepatic maturity of stem cell-derived HLC. Unfortunately, a comprehensive review concluded that despite numerous efforts, the engraftment and/or proliferation of these cells have been proven to be very poor when compared to the transplantation of primary hepatocyte, and unsuccessful to reach full liver humanization.[141] Conceivably, this could be attributed to incomplete maturation of the generated cells and the idea of "HLC *in vivo* maturation" might yield better results when further improvements are achieved *in vitro* before transplantation. The list of applications using humanized mouse models is extended beyond the aforementioned. They have been broadly used for the investigation of infectious diseases, such as malaria or hepatitis[142,143], and have provided insights into drug toxicity[144] and lipoprotein metabolism[145]. Noteworthy, the term "humanization" is frequently used for experimental animals that bear sequences of human DNA, such as mice carrying genomic sequences encoding the human A1AT[146] or apoE isoforms[147]. These models might satisfy the research needs in some circumstances, but in my opinion not to the same degree as liver-repopulated mice do. Finally, it is worth mentioning that other animal models of FAH deficiency exist, such as rats[148] and pigs[149], but they are not described or used in this thesis.

1.5 GENOME ENGINEERING

1.5.1 The beginning

Genome editing, often referred to as genome engineering, is targeted and specific genetic modification in living cells and has applications in a broad range of fields, including medical research and biotechnology. The fist documentation of genome engineering was performed by Hinnen and associates who succeeded to deliver genetic material through a plasmid into the yeast genome.[150] The foreign DNA sequence was commonly incorporated where the native gene was located, and less frequently at random *loci*; an observation that led to the conclusion that homology must play an essential role. The assumption was confirmed a few years later when it was shown that homology arms of foreign DNA are highly recombinogenic, and the editing efficiency increases 10- to 10,000 fold when the *locus* intended to be targeted is cleaved with a restriction enzyme generating double strand breaks.[151]

Spontaneous recombination was demonstrated in mammalian cells *albeit* at very low frequencies. Specifically, Smithies et al. attempted the integration of neomycin-expressing cassette in the β-globin *locus*, both in mouse and human cells, reporting an efficiency of 4 in 10^6 transfected cells.[152] Two years later, Capecchi et al. conducted similar experiments using microinjection instead of transfection showing editing efficiency of 1 in 10^3 cells.[153] The same groups achieved gene editing in mouse embryonic stem cells, a milestone that revolutionised the field of mouse genetics.[154,155]

Spontaneous recombination is characterized by limitations, such as low efficiency, as well as potential integration in random genomic sites. This hurdle can be partly overcome by inducing double strand breaks at the *locus* of interest, as shown in yeast[156] and later in mammalian cells[157]. The DNA lesions can be induced with various methods and cells have evolved diverse mechanisms to repair them. Among the most studied are HDR and non-homologous end-joining (NHEJ), which are schematically illustrated in Figure 6.

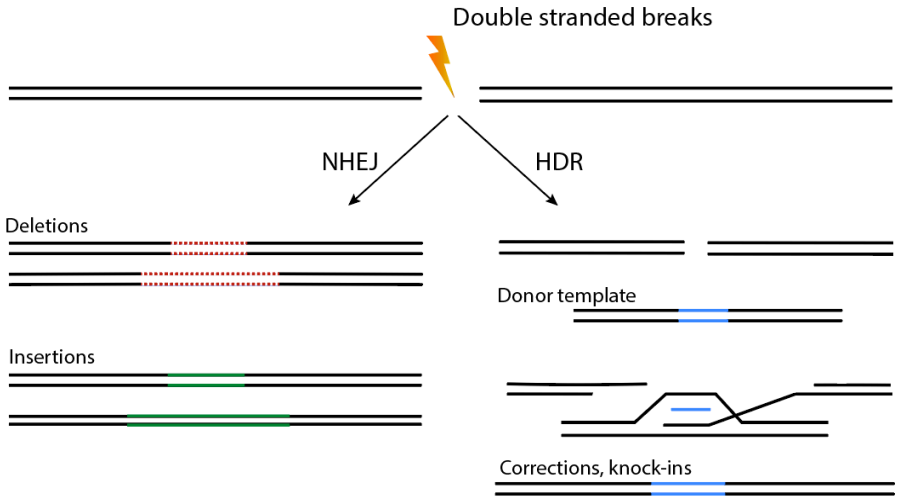

Figure 6: Cellular repair pathways of double strand DNA breaks. NHEJ: non-homologous end joining; HDR: homology-directed repair.

When NHEJ pathway occurs, the free ends of DNA are ligated randomly which is error prone, meaning that random insertions, deletions, substitutions or translocations of sequences of variable length are introduced at the *locus* of double strand break.[158] On the other hand, HDR is an error-free repair mechanism where the sister chromatid or homologous chromosome is used as a template. Therefore, this repair pathway occurs when cells undergo DNA replication, while NHEJ is active throughout the whole cell cycle. Practically, when genome engineering is applied, gene knockouts can be achieved through NHEJ, since the insertions/deletions in coding region often cause a shift of the reading frame and alter the protein translation or create a premature stop codon. On the contrary, precise modification or knock-ins can only be achieved through HDR and by providing a DNA donor template. In conclusion, the awareness that double strand breaks enhance precise genome editing advanced the development of programmable nucleases that would completely alter the entire field and would open new horizons in the coming years.

1.5.2 Programmable nucleases

Various molecules have been identified as potential candidates to mediate double strand breaks.[159-161] However, programmable nucleases, that can introduce single or double strand breaks by cleaving phosphodiester bonds between nucleotides, are proven to be the most efficient. The first programmable nuclease to be used was a zinc-finger nuclease (ZFNs).[162] Later, transcription activator-like effector nucleases (TALEN)[163] and CRISPR[164-166] were described and developed. A common characteristic of the above is the ability to target a specific genomic *locus* and induce DNA lesions, which are later corrected in a targeted manner. All three have unique features, but CRISPR dominates the field mainly because of its easiness in application and lower cost. Other useful tools for genome engineering are meganucleases, transposons, recombinases, as well as chemical methods. For the purpose of this literature review, focus will be given on CRISPR technology only.

1.5.3 CRISPR development and function

CRISPR system was first identified in *E. coli* in 1987 by a group of Japanese researchers who fortuitously cloned a series of short sequences interspaced by repeats. With the limited DNA sequence data and technology at that time, these arrays were not further investigated and remained a mystery.[167] Later in the early 2000s, the same pattern was reported in other bacteria strains, as well as some archaea.[168] Milestone studies in the CRISPR development showed that the sequences, called spacers, surrounded by repeat sequences are of viral or plasmid origin, leading to the conclusion that CRISPR is involved in the bacterial adaptive immune system.[169-171] This was confirmed in 2007, when spacers of different bacteriophage origins were incorporated or eliminated from the bacterial genome of a number of strains altering in this way their defence ability.[172] In 2010, it was shown that the nuclease involved in the CRISPR system, CRISPR-associated 9 (Cas9), could cleave bacteriophage and plasmid DNA at the spacer-specific region.[173]

With every viral infection of bacteria, a short new fragment of DNA of viral origin is incorporated in the CRISPR array, which is later transcribed and processed into mature RNA, named CRISPR RNA (crRNA), and serves as "guide" to direct the Cas9 nuclease for invading phage distruction.[174] Another necessary component of the system is the trans-activating crRNA (tracrRNA), a sequence in the CRISPR operon, which plays a role in the maturation of crRNA by ribonuclease II and the activation of mature crRNA by forming a duplex crRNA:tracrRNA.[175] A landmark for the CRISPR development occurred in 2012, when it was shown that the duplex is the factor that guides the nuclease to the DNA sequence, and it was proposed for the first time as "an alternative methodology based on RNA-programmed Cas9 that could offer considerable potential for gene-targeting and genome editing applications".[176] Shortly after that, three independent studies were published almost simultaneously, where CRISPR was applied to edit mammalian cells.[164-166] Specifically, human codon-optimized *S. pyogenes* Cas9[164-166] or *S. thermophiles* Cas9[164] were used along with the duplex crRNA-tracrRNA (commonly referred to as "guide RNA") and edited human cancer cell lines and iPSC[164-166], as well as murine cells[164]. A significant body of research work followed identifying other CRISPR nuclease orthologs[164,177,178], improving the specificity by modifying properties of guide RNA[179,180] or nuclease[181-183], as well as expanding the applications of the technology. Beside gene editing, CRISPR has been used for transcriptional[184] and epigenetic modifications[185], live imaging[186], large scale screenings[187], as well as microRNA[188] and mRNA modifications[189].
CRISPR technology has been characterized as "the greatest biological breakthrough since that of Francis Crick, James Watson and Rosalind Franklin" (The Guardian)[190], "the biggest game-changer to hit biology since PCR" (Nature Journal)[191], as well as has been proclaimed "breakthrough of the year" in 2015 (Science Journal)[192]. Later in 2020, the Nobel Prize in Chemistry was awarded to Emmanuelle Charpentier and Jennifer A. Doudna for the development of CRISPR. The power, versatility and robustness of this technique that has dominated the field is reflected by the explosion of scientific studies utilizing it. To illustrate this with numbers, a search on PubMed with the term "CRISPR" as query gave 6,138 results for the year 2020, which in other words would be 16-17 papers per day. Selected preclinical studies targeting liver diseases are described in the next sections.

1.5.4 Genome engineering in basic and translational hepatology research

1.5.4.1 In vitro gene editing

Since the milestone of editing mammalian cells with CRISPR technology[164-166], a wide range of cell types have been edited *in vitro* either to provide proof of concept or to generate isogenic cells in order to study various diseases or investigate potential sources for cell therapy. The majority of these liver-directed studies are performed on iPSC and liver cancer cell lines.
The first report on the generation of UCD-specific iPSC and their subsequent CRISPR-mediated correction was from Lee et al. who genetically corrected ARG1 deficiency in iPSC from multiple patients through the insertion of human *ARG1* cDNA. Correction was verified

on molecular level and enzyme activity measured both in iPSC and HLC.[90] Another proof-of-concept study on ARG1 deficiency used CRISPR in combination with *piggyBac* to restore *Arg1* deficiency in mouse iPSC. Even though the editing was excellently demonstrated on molecular level, minimal urea cycle function was shown due to hepatic immaturity of the iPSC-derived HLC.[193]

A1AT and hemophilia are among popular liver genetic disorder targets for genome engineering. In a recent report, scientists generated iPSC from 28 A1AT deficient patients with various mutations and made them open to sharing with the research community. In this study, iPSC from two donors were corrected with CRISPR technology, differentiated into HLC and phenotypically characterized, with the corrected HLC showing less intracellular, aggregated A1AT protein and increased secreted protein.[194] IPSC models of hemophilia have been also made and corrected with CRISPR platforms by reverting inversions[195] or applying more universal approaches[196].

Other *in vitro* liver-related applications of CRISPR were mainly in the field cancer where the system was used to delete genomic regions using two gRNAs in close proximity to each other[197] or knock-out genes as tools to study hepatocellular carcinoma[198,199]. Furthermore, CRISPR has been applied to generate reporter cell lines for various purposes[200] or inhibit HCV in hepatocellular carcinoma cell lines[201].

1.5.4.2 In vivo gene editing

The first *in vivo* CRISPR-mediated liver-targeted study was in a mouse model of hereditary tyrosinemia type I (HT-I), a liver disease caused by mutations in the *Fah* gene. The delivery of CRISPR/Cas9 system through hydrodynamic injection corrected 0.4% of liver cells, which expanded to 33% because of the growth advantage that Fah proficient cells display over the deficient ones.[202] While this level of correction was sufficient to correct FAH deficiency, the results were limited by the type of delivery method used which is unlikely to be applicable in a clinical setting. Later, same researchers delivered the editing reagents with combination of lipid nanoparticles and AAV reporting *Fah* gene correction rates of 6%.[203] Another proposed strategy for the treatment of HT-I was "metabolic pathway reprogramming" which interrupts metabolic pathways that lead to accumulation of toxic compounds due to gene deficiency, and reroutes them to non-toxic pathways leading to a more benign phenotype. Characteristically, HT type I caused by mutations in *Fah* gene, was converted into HT type III which has a milder phenotype, by loss of function of *Hpd* gene that acts upstream of *Fah*. The dual gRNA and nuclease effectively inactivated approximately 8% of hepatocytes following hydrodynamic injection in $Fah^{-/-}$ mice. After the course of 2 months, hepatocytes mutated for both *Fah* and *Hpd* fully reconstituted the mouse livers making the animals healthy and asymptomatic.[204]

OTCD, the most common UCD, has been also a target of *in vivo* genome engineering. Specifically, a dual AAV system was used to express the CRISPR components and administered via tail-vein injection into spf^{ash} mice, a mouse model of the deficiency. The study showed correction in around 10% of hepatocytes in neonates and improved survival after being challenged with high-protein diet. Importantly, the therapeutic benefit in adult animals was much less notable (<1.7%).[205] The difference in the effect between neonatal and

adult animals could be predicted since the high cell proliferation in neonatal livers would favour HDR-based repair, while hepatocytes in the adult animals, that are mostly quiescent, are very likely to undergo NHEJ. The indels introduced by the latter repair mechanism diminished residual enzyme activity exacerbating the disease phenotype in adults. This observation should be taken into consideration if applied in compound heterozygotes where undesired NHEJ editing might generate more deleterious mutations. Another report targeting OTCD used patient derived hepatocytes xenografted in FRG mice with successful correction in up to 29% of alleles. Investigators concluded that if such approach was recapitulated in humans, it might yield clinical benefits, given the fact that several obstacles will be overcome, including efficiency of delivery, cell proliferation required for homologous recombination and the risk of converting alleles with residual activity into null.[138] Admittedly, the results of this study were very encouraging; yet, I consider that except from the excellent molecular correction (deep sequencing and immunohistochemistry), phenotypic investigation and characterization would be of great value.

CRISPR technology has been utilized in an attempt to treat haemophilias which are X-linked haemorrhagic disorders caused by defective genes encoding coagulation factors. AAV-mediated gene knock-in of coagulation factor VIII showed 0.2%-0.3% editing efficiency four weeks after treatment which correspond to approximately 7% normal human plasma activity levels. This increased to around 13% after two months, which is above the level that patients would display a clinical benefit.[206] Other similar studies administered the CRISPR reagents either with plasmid vectors through hydrodynamic injection or with AAV vectors reporting gene editing in the range of 0.5-1.5%. These levels of correction ameliorated the phenotype but did not reverse it completely.[207,208] A noteworthy observation in one of the studies above was that while naked DNA constructs displayed correction rates of 0.56% of DNA in hepatocytes and were adequate to restore homeostasis, AAV-mediated administration gave higher editing efficiency but with no therapeutic effect perhaps due to severe hepatotoxicity, according to the investigators.[208]

Recently, AAV vectors have been the preferred method of administration for *in vivo* genome editing studies. Despite the encouraging results in many instances, their use has been associated with hepatic injury and immunogenicity.[209] Therefore, other delivery options are explored with lipid nanoparticles having gained significant popularity. The anionic nature of nucleic acid prohibits the entrance to the cell through the cell membrane. Their encapsulation into cationic lipid nanoparticles makes the delivery relatively easy. Another appealing aspect of lipid nanoparticles is that they do not contain any viral component which minimizes the risk of immunogenicity. Additionally, the presence of delivered DNA/RNA/protein with lipid nanoparticles is transient; thus, limiting the potential of off-target events, as well as repeated dosing is possible in order to achieve relevant level or editing, and consequently sufficient therapeutic effect. A study solely used lipid nanoparticles for the delivery of sgRNA and nuclease in order to knock-out the transthyretin (*Ttr*) gene in mice achieving the impressive level of 70% editing efficiency of liver cells which led to > 97% decrease of TTR protein in serum.[210] It is worth noting that this study used RNA for Cas9 nuclease and gRNA delivery; however, the encapsulation of reagents into lipid nanoparticles might prove troublesome if combination of different molecules to be delivered is required (*e.g.* DNA for the donor template, protein for Cas9 nuclease).

An obstacle for precise correction of hepatocytes *in vivo* is their quiescent nature. The main repair pathways of double strand DNA breaks (Figure 6) work in parallel in a single cell.

HDR is predominantly active during S and G2/M phases and NHEJ during G0/G1. This implies that non-dividing cells, like the majority of hepatocyte in an adult liver, would favour the latter repair mechanism making gene knock-outs less challenging than precise manipulation that rely on HDR. Therefore, a number of studies present benefit from such approaches, many of those focusing on lipid and lipoprotein metabolism.

PCSK9 degrades low density lipoprotein (LDL) receptor controlling indirectly LDL serum levels, a well-established risk factor for cardiovascular diseases.[211] Based on this knowledge, investigators sought to knock-out the gene proposing it as a way to prevent cardiovascular diseases in humans. An initial study achieved nearly 50% gene disruption in the mouse liver resulting in 35-40% decrease of plasma cholesterol.[212] Same researchers conducted a follow-up study employing liver-humanized mice reporting similar editing efficiency, 50% reduction of blood PCSK9; yet, they failed to significantly reduce plasma cholesterol perhaps due to compensatory effects from residual mouse cells in the liver, as explained by the authors.[137] Indeed, a "one-time treatment" might sound attractive; however, it is quite unlikely that this might replace the statins because the alterations are permanent and they cannot be withdrawn in case that side-effects occur.

Disruption of genes has been used in several studies to establish disease models, such as that of familiar hypocholesterolaemia by disrupting the LDL receptor gene (*Ldlr*) in mice.[213] Almost complete reduction of LDLR protein in the liver was achieved which caused hypercholesterolemia and atherosclerotic plaques. In a similar fashion, CRISPR-mediated knock-outs have been applied in somatic cells to generate models of hepatocellular carcinoma. Common targets for such applications are tumour suppressors *Pten* and *p53*.[214,215] Altogether, this scientific evidence demonstrates the relative simplicity to generate models of liver cancer and overpass the embryonic stem cell targeting stage. Finally, CRISPR has been used *in vivo* to combat viral infections, mainly HBV and HCV either by deleting viral DNA or inhibiting the expression of viral protein or the entry in the cells.[216]

A landmark in genome engineering is the development of base editors which install point mutations without the reliance on HDR, consequently negating the need for actively dividing cells. These are produced by the combination of catalytically impaired CRISPR nucleases, making them unable to induce double strand DNA breaks, and fusion of DNA deaminases. The cytosine base editors (CBE) were initially generated which catalyse the conversion of cytosine to guanine.[217] Later the adenine base editors (ABE), which convert adenine into thymine, were developed.[218] Jointly, they could be used to accommodate all possible nucleotide conversions which would be applicable to approximately 30% of current annotated pathogenic variants.[219] The percentage of the targetable spectrum of disease-causing variants was further increased with the development of prime editors which consist of nickase Cas9 fused to an engineered reverse transcriptase enzyme that permit the introduction of all possible point mutations conversions, as well as small insertions or deletions.[220] The applicability of base editors has been further maximized with constant research on other Cas9 orthologs, different PAM sequences or modification of editing window features.[221] Base editors are considered superior to conventional CRISPR nucleases because of their independence on cell proliferation, as well as their mode of function without inducing double strand breaks which make them safer. The technology has been applied to target genes in various types of somatic cells and embryos, as well as organisms (mice, rabbit, zebrafish, pigs etc.)[221]; several of those are liver-directed including phenylalanine hydroxylase (*Pah), Fah* and *Pcsk9* and are briefly described below.

Phenylketonuria (PKU) is a liver metabolic disorder occurring in case of defective *PAH* gene and results in hyperphenylalaninemia due to decreased metabolism of phenylalanine. Clinical manifestations include severe retardation, microcephaly, and seizures.[222] *Pah*^{enu2}, a mouse model that recapitulates the disease phenotype, has been used in an elegant study where investigators provided evidence that AAV-mediated base editing successfully rescued the phenotype.[223] Specifically, correction rates on DNA level were reported to be 25.1%, while on mRNA level 43.6% evaluated 26 weeks post treatment. The discrepancy of efficiencies between DNA and mRNA was explained by the fact that nonparenchymal cells account for 30-40% of total liver cell population and *Pah* gene is expressed only in hepatocytes. Furthermore, edited mice displayed increased PAH enzyme activity, improved weight and changed the fur colour indicating the phenotypic restoration. Another study that investigated the potentials of base editing in a mouse model of tyrosinemia (*Fah* deficiency) through hydrodynamic injection showed 9.5% efficient base substitution 5 days post administration.[224] Investigators stated rescue of weight loss and presence of wide-spread patches of FAH-positive hepatocytes after 32 days of monitoring. Unfortunately, there was no quantification of FAH positivity in the liver or other phenotypic characterization. Finally, there have been other liver-directed studies utilizing base editing *in vivo*, with *Pcsk9* gene being among the most popular targets and the reported efficiencies of precise genomic alteration ranged between 10-40%.[225-227]

1.5.4.3 Ex vivo gene editing

Another option for gene editing might be the genetic manipulation of cells *ex vivo*. This implies the isolation/extraction of cell population of interest from the patient, genetic alteration outside of the body and reinfusion back into the patient. *Ex vivo* gene editing has several advantages over the *in vivo* approach, including wider variety of delivery means, ability to analyse and enrich the edited cells, as well as better control over the amount of delivered components, which is a crucial factor since the magnitude and duration of the nuclease expression is positively associated with unspecific mutagenesis.[228]

Some cell populations, such as those belonging to the hematopoietic system, including hematopoietic stem cells and primary lymphocytes, are easier targets for *ex vivo* gene editing.[229,230] These cell types are already underway in research and clinical applications, not only because they are easier to work with, but also because of the extensive efforts on isolation, culture and transplantation of these cells. Furthermore, there is considerable experience with gene therapy with these cell types; consequently, protocols and techniques have been well established.

Liver-directed *ex vivo* gene editing presents many hurdles and is more challenging compared to the manipulation of blood or immune cells. First, hepatocytes cannot survive and maintain their functions *in vitro* for a prolonged period of time. Secondly, despite a lot of optimizations on hepatocyte isolation, the viability and quality of the isolated cells differ greatly depending on the isolation procedure and the donor. Third, transplantation and engraftment capacity are not optimal, which means that in order to have a clinical benefit, a large number of hepatocytes would be required and repeated transplantations would likely be necessary. In response to this, generation of large numbers of human hepatocytes in live bioreactors has

been proposed[231], particularly pigs which share size, anatomy and biological similarities with humans[232]. However, concerns with zoonosis might impede the occurrence of this scenario.
Another critical obstacle in correcting primary hepatocytes *in vitro* is their quiescent nature which hampers the precise genetic corrections through HDR with conventional CRISPR technologies. To this end, the development of the aforementioned base editors[217,218] might assist in overcoming this hurdle. Furthermore, some conditions might benefit from gene knockouts or random NHEJ-based insertions/deletions, which are less challenging.
Another impediment for *ex vivo* editing of primary hepatocytes is the fact that they are not easily amenable to cell sorting that would make possible, in some cases, the enrichment of the successfully edited cells. Importantly, efficacy would be disease-specific and depend on the ability of the cells to expand, as well as the fitness profile of the edited cells over the unedited counterparts. Therefore, diseases such as A1AT deficiency, progressive familial intrahepatic cholestasis or Wilson's disease might be suitable candidates.[233-235] Finally, a successful *ex vivo* gene editing of primary hepatocytes would be dictated by the gene correction threshold that would yield a therapeutic benefit. For example, diseases such as UCD would most probably benefit from over 15% edited cells in order to clear the body from the excess nitrogen, while patients with hemophilia B would have a mild phenotype of the disease with only 5% of normal levels of factor IX disease[236].

1.5.5 CRISPR in clinical trials

CRISPR is an emerging gene editing tool rapidly progressing into clinical trials, with the currently reported ones being on Phase I or II stages. The vast majority of initiated clinical trials are based on *ex vivo* engineering of the cell population, followed by their administration back into the patient, and are mainly applied for haematological diseases and cancers. Among targets of blood diseases are beta thalassemia (NCT03655678, NCT03728322) sickle cell disease (GPH101, EDIT-301, NCT03745287) and human immunodeficient virus infection (NCT03164135), while many pharmaceutical and academic institutions are investigating treatments of B- or T-cell malignancies (B-cells: NCT04037566, NCT03398967, NCT03166878, NCT04637763, 2018-003916-38, NCT04557436, NCT04629729, NCT04035434, NCT02863913, NCT03399448, NCT04244656, 2019-003462-40; T-cells: NCT03690011, NCT04502446). Furthermore, a number of trials focus on T-cell engineering as an attempt to treat solid tumours, such as hepatocellular (NCT04417764), nasopharyngeal (NCT03044743) or renal (NCT04438083) carcinomas, as well as oesophageal (NCT03081715) and lung (NCT02793856) cancers.
When it comes to the use of CRISPR *in vivo*, there are only three clinical trials reported at the moment, two directed to eye-related pathologies (Herpes Simplex virus refractory keratitis NCT04560790 and Leber congenital amaurosis NCT03872479), while another to HPV-related cervical neoplasia (NCT03057912). Perhaps, the main issue limiting the *in vivo* use is the unavailability of target-specific and efficient delivery of editing agents.
So far, there are no reports of *in vivo* CRISPR-based clinical trials investigating potential genetic treatments of hepatic pathologies, other than familial transthyretin amyloidosis (NCT04601051). Familial transthyretin amyloidosis is not considered *per se* a liver disorder because it primarily affects other body's tissues and organs caused by the build-up of amyloid

protein, but the defectively encoded gene (Transthyretin, *TTR*) is primarily expressed in the liver and brain, and liver transplantation is considered the main treatment for the condition. Unprecedented interest in the technology has been showed from the pharmaceutical sector. Their pipelines include some liver-related genetic conditions, such as glycogen storage disease (CRISPR Therapeutics and Beam Therapeutics), A1AT deficiency (Intellia Therapeutics and Beam Therapeutics), as well as haemophilia A and B (Intellia Therapeutics), all of them being on research stage without having proceeded to clinical trials. Finally, beyond editing application, CRISPR holds great promise in the field of diagnostics which was revolutionised by the discovery and characterization of Cas13a (formerly C2c2) and Cas12a (formerly Cpf1), and their consequent introduction as diagnostic tools.[237,238] Currently, striking examples are diagnostic kits developed for combating the ongoing COVID (COrona VIrus Disease) pandemic from Mammoth Biosciences and SHERLOCK Biosciences. Other CRISPR-based tools were introduced for the diagnosis of Human enterovirus infection (NCT04535648) and Pertussis-Whooping Cough (NCT04535505).

1.6 OTHER POTENTIAL TREATMENTS FOR UREA CYCLE DEFECTS

Research is ongoing in the marathon for the identification of potential treatments for UCD, some of which are briefly discussed below.

Protein replacement therapy: Protein replacement therapy refers to a medical treatment that supplements or replaces an absent or deficient protein in a patient. This could be a potential treatment for lysosomal storage disorders because lysosomal enzymes are easily up-taken by the cells. The approach might be challenging in the case of UCD because cytosolic and mitochondrial enzymes are not naturally internalized by the cells. However, ARG1 deficiency might be a potential candidate for protein replacement therapy because except from hyperammonemia management, plasma arginine normalization is the consensus treatment goal for the disorder. The high arginine levels observed in affected patients could be depleted with protein infusion. Preclinical data on a mouse model of ARG1 deficiency showed reduction of plasma arginine levels after administration of recombinant arginase enzyme. On the contrary, ammonia levels were not normalized, perhaps due to inefficient entry of the protein into the hepatocytes.[239] A phase II clinical trial assessing the efficiency of such treatment in adult and paediatric patients is currently ongoing (NCT03378531).

mRNA therapy: Alternative to protein replacement therapy could be modified mRNA treatment which is highly promising for the management of various diseases, and advantageous over DNA- and protein-based therapies on several aspects. The use of mRNA is considered safer in regards to tumorigenicity and insertional mutagenesis. Delivery of mRNA could be facilitated through lipid nanoparticles which are very stable and immunological inactive.[240] Furthermore, liver is considered an easy target as it presents exceptional incorporation of lipid nanoparticles. A preclinical report normalized plasma ammonia and urinary orotic acid levels, along with improved survival rates in an OTCD murine model.[241] In another study, *ARG1* mRNA was delivered to cells *in vitro* or administered to wild-type mice, and showed an increase in protein expression.[242] The therapeutic potential of mRNA was further enhanced when a similar study administered *ARG1* mRNA to *Arg1* deficient mice every 3 days and it showed 100% survival without signs of hyperammonemia or weight loss, while the control group did not survive beyond day 22.[243] Noteworthy, if such treatment proves clinically translatable, multiple repeated infusions would be necessary due to the short lifetime of mRNA in the cells increasing significantly the cost of the treatment.

Gene therapy: For more than two decades, gene therapy has been in the epicentre of research and discussions. Some scientists consider UCD good candidates because of their severe nature, as well as because liver is a relatively easy to target. A number of viral vectors have been explored, with AAV being the most attractive. AAV vectors have been applied in a gene therapy study on a heterozygous OTCD mouse model correcting the underlying metabolic abnormalities[244], and led to an ongoing phase I/II clinical trial in OTCD adults with late onset (NCT02991144). A previous phase I/II gene therapy clinical trial in an OTCD patient had unanticipated clinical sequelae and led to fatal acute toxicity[125], however adenoviral vectors were delivered in this study, not AAV.

Similar preclinical studies have been performed in murine models of other UCD. Specifically, neonatal AAV-mediated administration of *ASL* gene increased the survival of *Asl*$^{-/-}$ animals in a dose-specific manner, but failed to maintain the weight to an equivalent degree to the control group. According to the investigators, the inefficacy of the treatment to fully reverse the phenotype was attributed to vector dilution due to animal growth. Therefore, vector re-administration was suggested which might prove challenging because of existing neutralizing antibodies to the vector capsid triggered by the initial administration.[245] Reduced plasma ammonia and citrulline levels, as well as improved survival and growth was shown in a AAV-mediated gene therapy study on a murine model of citrullinemia type 1.[246] Except from AAV vectors, adenoviruses have been employed as delivery means to target ASS1 deficiency in mice.[117,118] Finally in another report, ARG1 deficiency phenotype was to a great extent rescued in a murine model of the disease after AAV-based gene therapy.[247]

Among all UCD, ASL and CPS1 deficiencies are considered more difficult targets. The first mainly because the gene is expressed in several tissues (brain, liver, kidney, gut and arteries); therefore, it would be expected that a liver-directed gene therapy would show improvements of urea cycle activity in the liver, but not the other aspects of the phenotype.[248] Regarding CPS1D, packaging of the transgene into the viral vector is considered troublesome due to the large length of the gene. To overcome this, the transgene could be split into halves but then the difficulty is that each cell needs to receive both of the half copies such that, when combined, they could be translated into the CPS1 protein.[249] A study applied this approach and packed *CPS1* transgene and its regulatory cassettes into two separate vectors.[250] In this report, treated mice had longer survival rates, as well as improved ammonia, CPS1 expression and activity. Finally, except from packaging limitations, other drawbacks and challenges of gene therapy worth mentioning are inadequate transgene expression, genotoxicity, vector dilution with cell proliferation and the requirement for vector re-administrations which is hindered by induction of immune responses.[251]

2 RESEARCH AIMS

The overall aim of this thesis was to investigate liver genetic disorders in the context of disease modelling and treatment through genome engineering. Both objectives were investigated *in vitro* in iPSC or primary hepatocytes, as well as *in vivo*, in a liver-humanized mouse model. The thesis focusses specifically on UCD; however, the described strategies could be undoubtedly be tailored to other liver genetic defects.

Specific aims of the thesis:

PAPER I *Title:* Applying hydrodynamic pressure to efficiently generate induced pluripotent stem cells via reprogramming of centenarian skin fibroblasts.
Aim: to improve somatic cell reprogramming and generate patient specific iPSC.

PAPER II *Title:* Guide to the assessment of mature liver gene expression in stem cell-derived hepatocytes.
Aim: to generate a gene expression profile dataset of human primary liver tissues and investigate the hepatic maturation of stem cell-derived HLC.

PAPER III *Title:* Gene editing correction of a urea cycle defect in organoid stem cell derived hepatocytes.
Aim: to generate an *in vitro* disease model platform of UCD and genetically correct the defective gene.

PAPER IV *Title:* A liver-humanized mouse model of carbamoyl phosphate synthetase 1 deficiency.
Aim: to generate a liver-humanized mouse model of CPS1D and determine if it faithfully recreates the human disease.

PAPER V *Title*: Correction of a urea cycle defect after *ex vivo* gene editing of human hepatocytes
Aim: to generate a liver-humanized mouse model of OTCD and assess efficacy and safety of *ex vivo* gene editing of primary human hepatocytes, *in vitro* and *in vivo*.

3 MATERIALS AND METHODS

Materials and methods used for the research work of this thesis are only briefly described in this section. For more details regarding protocols and procedures, readers are redirected to the constituent papers.

3.1 ETHICAL CONSIDERATIONS

PAPER I was produced within the framework of HUMAN project. Centenarian fibroblasts were obtained from DIMES Department of University of Bologna, Italy, with Institutional Review Board Approval (Prot. n. 2006061707 and Prot. n. 79/2015/U/Tess). Fetal and adult (postnatal) liver tissues were isolated at the University of Pittsburgh under approvals PR010020037 and IRB 0411142, and were used in **PAPERS II** and **III**. Human primary hepatocytes isolated at Karolinska Institutet under ethical approval Dnr. 2010/678-31/3 were used in **PAPERS III-V**. Ethical approval ID400 42-17 allowed the animal experiments in **PAPERS IV** and **V**.

3.2 CELL CULTURE

Fibroblasts in all studies were cultured in fibroblast medium which consisted of DMEM-GlutaMax supplemented with 10% heat-inactivated fetal bovine serum, 1mM non-essential amino acids and 1% penicillin/streptomycin (all from Thermo Fisher). IPSC were cultured in Essential 8 medium (Thermo Fisher) on vitronectin (Thermo Fisher) coated dishes or plates. Colonies of iPSC were mechanically passaged, while when iPSC were as single cells, they were passaged with Accutase (Sigma Aldrich) every 4-5 days. Primary hepatocytes were cultured in Hepatocyte Maintenance Medium (HMM) medium (Lonza) on collagen coated plates.

3.3 IPSC GENERATION AND CHARACTERIZATION

3.3.1 Somatic cell reprogramming

Dermal fibroblasts in **PAPER I** and liver fibroblasts in **PAPERS II-IV** were reprogrammed with CytoTune 2.0 Sendai Reprogramming Kit (Thermo Fisher), delivering the Yamanaka factors.[37] Modifications to manufacturer's protocol were applied as described in **PAPER I**.

3.3.2 Characterization of iPSC clones

Pluripotency of iPSC clones generated in **PAPERS I-III** and **V** was assessed with various immune-based (flow cytometry, immunocytochemistry, alkaline phosphatase) and molecular techniques (quantitative polymerase chain reaction) that are described in the respective sections below. PluriTest[252,253] was additionally used as a tool for pluripotency assessment in **PAPERS I** and **III**. The assay was carried out entirely at the Mutation Analysis Facility (Karolinska Institutet) and sequencing data was analysed with the bioinformatic tool provided at www.pluritest.org.

3.4 IMMUNO-BASED TECHNIQUES

3.4.1 Flow cytometry

Flow cytometry was applied to evaluate pluripotency of generated iPSC clones in **PAPERS I** and **III**. Briefly, iPSC were dissociated with Accutase (Sigma Aldrich) and washed with PBS. Thereafter, cells were stained for 20 min at room temperature with Live Dead Violet Kit (Thermo Fisher), and surface antigen conjugated antibodies: BV605-conjugated mouse anti-TRA1-60 (563187), Alexa 647-conjugated mouse anti-TRA1-81 (560793), PE-CF594-conjugated mouse anti-SSEA1(562485), PE-conjugated rat anti-SSEA3 (560237), (all from Becton Dickinson). For nuclear staining, fixation and permealization was performed with Fix/Perm Buffer Set (Thermo Fisher) according to manufacturer's instructions, following incubation with PE-conjugated anti-NANOG (560483), PerCP-Cy5.5-conjugated anti-OCT3/4 (560794), Alexa 647-conjugated anti-SOX2 (560302), (all from Becton Dickinson). Flow cytometry was carried out with a LSR Fortessa Analyzer (Becton Dickinson) and data was analysed with FCS Express 4 (De Novo Software) having appropriate isotype controls for gate setting.

3.4.2 Fluorescence activated cell sorting (FACS)

FACS was used in order to enrich the positively transfected cells based on GFP positivity in **PAPER III**. More details are provided in the section - Genome editing of iPSC.

3.4.3 Immunocytochemistry (ICC)

In **PAPER I**, ICC served for pluripotency assessment of iPSC. Briefly, cells were pre-incubated in PBS with 10% FBS for one hour at room temperature. Thereafter, they were washed three times with PBS and incubated for one hour at room temperature with primary antibodies anti-OCT4 (MA1-104); anti-SSEA4 (MA1-021); anti-TRA1-60 (MA1-023); anti-TRA1-81 (41–1100), (all from Thermo Fisher). Next, cells were washed with PBS and incubated with Alexa-488 (A-11001) or Alexa-594 (A-11005) goat anti-mouse IgG (Thermo Fisher) for 1 hour at room temperature. Finally, nuclei were counterstained for 2 min with DAPI (Thermo Fisher) and visualized with a fluorescence microscope (Olympus iX73).

3.4.4 Immunohistochemistry (IHC)

In **PAPER V**, IHC analysis was performed on liver tissues of humanized mice. In short, liver sections from repopulated animals with unedited OTCD, edited OTCD or OTCP hepatocytes were collected, fixed in 4% buffered formalin, embedded in paraffin and sectioned to a 4 μm thickness. Human-specific antibodies for anti-cytokeratins 8 and 18 (clone 5D3, dilution 1:100, Thermo Fisher) or anti-OTC (HPA000243, dilution 1:400; Sigma Aldrich) were used. Tissues were additionally counterstained with haematoxylin. Pictures were analysed with Cleans Dimension 1.12 software.

3.4.5 Enzyme-linked immunosorbent assay (ELISA)

Human albumin was quantified either in sera of liver-repopulated animals (**PAPERS IV** and **V**) or in culture media from iPSC-HLC (**PAPER III**). In mice, human albumin concentrations were used to estimate the level of liver humanization. From previous studies[130,133], it is well known that each 1 mg/ml circulating human albumin corresponds to approximately 20% liver humanization. Albumin was measured every 2 weeks starting from

4^{th}-5^{th} week post transplantation with Human Albumin Elisa Quantitation Kit (Bethyl Laboratories). In **PAPER III**, media from genetically unedited and edited iPSC-HLC were collected on days 20 and 26 of hepatic differentiation protocol and quantified with the same kit above. Human A1AT was also measured in **PAPER V** in mouse sera with Human alpha 1 Antitrypsin ELISA Kit (SERPINA1) (abcam) according to manufacturer's instructions.

3.5 MOLECULAR TECHNIQUES

3.5.1 Nucleic acid isolation

Throughout all studies, DNA was isolated using DNeasy Blood & Tissue Kit (Qiagen) following manufacturer's instructions. RNA was isolated either using the Pure Link RNA Mini Kit (Thermo Fisher) or TRIzol reagent (Thermo Fisher). Amounts of nucleic acids were quantified with Nanodrop Spectrophotometer (Thermo Fisher).

3.5.2 Polymerase chain reaction (PCR)

PCR was used for various purposes within this thesis. Information on primers, enzymes and amplification conditions are provided in the respective sections (*e.g. OTC* transcript amplification, Estimation of editing efficiency, Editing quantification, Restriction enzyme digestion and analysis of DNA fragments etc.).

3.5.3 Complementary DNA (cDNA) synthesis

In all studies cDNA was synthesized using High Capacity Reverse Transcriptase Kit (Thermo Fisher) according to manufacturer's instructions. The produced cDNA was diluted 10x with RNAse free water and consequently used for qPCR.

3.5.4 Quantitative polymerase chain reaction (qPCR)

Each reaction for qPCR consisted of 2 µL cDNA input, 5 µL TaqMan Universal PCR Master Mix (Applied Biosystems), 0.5 µL TaqMan assay (Thermo Fisher) and 2.5 µL water. All TaqMan assays used are reported in **PAPERS I-V**. Reactions were run in duplicates or triplicates using StepOnePlus Thermocycle (Applied Biosystems). In all experiments, human cyclophilin A (*PPIA*) mRNA was utilized as endogenous control. Further information regarding the thermocycle parameters, calculations and data representation is described in **PAPER II**.

3.5.5 Molecular karyotyping

Molecular karyotypic analysis was entirely performed at ATLAS Biolabs (Germany) using Affymetrix CytoScan HD Array. Software Chromosome Analysis Suite (ChAS 4.0) was used to analyse Cyhd.cychp files.

3.5.6 Gel electrophoresis

RNA or DNA samples in **PAPERS II-V** were run on agarose (Sigma Aldrich) gel containing GelRed Nucleic Acid Stain (Biotium).

3.5.7 Disease-causing variant identification

In **PAPER III**, the disease-causing variant was identified applying long range PCR with PrimeStar GLX DNA polymerase (Takara) following manufacturer's instructions. Eight

regions were amplified in order to cover the whole *OTC* gene, both introns and exons. Information regarding the sequences of the primers are provided in the respective paper. Amplicons were pooled and target sequenced by Macrogen (Seoul, South Korea); (Run type 100bp PE, platform HiSeq2000, Shotgun library type, TruSeq DNA PCR free 350bp). Reads from sequencing data were aligned to reference *OTC* gene on NCBI using the Geneious 8.1.9 software.

In **PAPER V**, A different approach was applied to identify the mutation causing the OTCD. Specifically, RNA from patient hepatocytes was reverse transcribed with Prime Script Reverse Transcriptase kit (Takara) according to manufacturer's instructions. Next, transcripts were amplified with Pfu Ultra II Fusion HS DNA polymerase (Agilent Technologies) from exon 1 to exon 5 with forward primer 5'-GAAGATGCTGTTTAATCTGAGG and reverse primer 5'-CTGGAGCGTGAGGTAATCAGCC, and from exon 5 to exon 10 with forward primer 5'-GCAGATGCAGTATTGGCTCG and reverse primer 5'-CCCATACCACGTGTTAGGGATT, as previously described.[254] The suspected genomic *locus* containing the mutation was amplified with forward primer 5'-TCTCATCCTCATGTCCAAAGTGTT and reverse primer 5'-TATGTAAAGCCACACCCACAGAC using Taq DNA polymerase (NEB). Finally, the amplicons were Sanger-sequenced (Macrogen) and analysed with Geneious 8.1.9 software.

3.5.8 *OTC* transcript amplification

OTC transcript amplification in **PAPERS III** and **V** was conducted as described in section - Disease-causing variant identification.

3.6 HEPATIC DIFFERENTIATION AND ORGANOID FORMATION

IPSC were differentiated into HLC organoids in **PAPERS II** and **III**. The protocol consisted of 4 stages (endoderm, hepatoblast, organoid and HLC) lasting 26-28 days in total. ActivinA was the main component for endoderm induction while BMP4 and bFGF for hepatoblast formation. Organoid were generated following previously described medium[255] applying modifications in our laboratory. Main components for hepatic differentiation were HGF, rifampicin, hydrocortisone 21-hemisuccinate sodium salt, dexamethasone and oncostatin M. Readers are redirected to **PAPERS II** and **III** for complete differentiation protocol.

3.7 CRISPR APPLICATION

3.7.1 Genome editing of iPSC

In **PAPER III**, iPSC were genetically modified in which a point mutation causing OTCD was targeted. Shortly, multiple gRNA were designed against the human *OTC* gene, and positioned as close as possible to the mutation. Vectors expressing wild-type or nickase Cas9 were used, in which a gRNA was cloned. Vectors were ordered from Addgene, while gRNA and DNA donor templates from Sigma Aldrich. After vector construction and verification of successful insertion of gRNA, transfection was performed with 4D-Nucleofector System (Lonza) with electroporation buffer P3 Primary Cell 4D-Nucleofector X Kit L (Lonza). Enrichment of successfully transfected cells based on GFP positivity was performed using fluorescence-activated cell sorting (FACS). After cell recovery and expansion, editing efficiency was estimated based on a restriction enzyme digestion assay which would cleave only DNA sequences that have correctly undergone the HDR-based repair pathway.

Moreover, efficiency was also validated with Synthego Performance Analysis Interference of CRISPR Edits (ICE, Synthego; https://ice.synthego.com). Finally, cells were clonally expanded and editing was evaluated in every clone with the same techniques described above. Genomic region of interest in the fully digested clone was also Sanger-sequenced (Macrogen).

3.7.2 Genome editing of primary hepatocytes

In **PAPER V**, human primary hepatocytes were genetically engineered. In short, the intronic region containing the disease-causing variant was deleted using a dual gRNA approach. Several pairs of gRNA (all from IDT) were tested upstream and downstream of the mutation. *Streptococcus pyogenes* Cas9 (*SpCas9*) enzyme was delivered to the cells as protein (IDT). Cells were transfected with 4D-Nucleofector System (Lonza) with electroporation buffer P3 Primary Cell 4D-Nucleofector X Kit L (Lonza) and program CA137. Some of the transfected cells were analysed *in vitro* for editing efficiency after 24-48h, while the remainder were immediately transplanted into the FRGN mice. The genomic region of interest was amplified and editing efficiency was estimated based on band intensities on agarose gel and corrected for the amplicon length. Readers are redirected to **PAPER V** for further information.

3.7.3 Off-target mutagenesis investigation

Two different approaches were used in order to investigate potential off-target mutagenesis that might have arose from CRISPR application. In iPSC (**PAPER III**) whole genome sequencing of unedited and edited iPSC clones was applied. Whole genome sequencing, as well as bioinformatic analysis was performed by Macrogen. CIRCLE-seq technique was applied in **PAPER V** in order to evaluate off-targets in edited primary hepatocytes, and was entirely carried out by collaborators at AstraZeneca. Further details regarding CIRCLE-seq are provided in **PAPER V**.

3.8 ^{15}N INCORPORATION INTO UREA

^{15}N incorporation into urea was utilized as a tool to measure urea production and restoration of urea cycle after genome engineering in iPSC (**PAPER III**) or primary hepatocytes (**PAPER V**). Specifically, cells were incubated with $^{15}NH_4Cl$ and ornithine. Media was collected in which labelled and unlabelled urea was quantified with mass spectrometry.

3.9 HEPATOCYTE TRANSPLANTATION

In **PAPERS IV** and **V**, the FRGN mouse model was used which is a triple knockout mouse (*Fah*$^{-/-}$, *Rag2*$^{-/-}$, and *IL2rg*$^{-/-}$) on NOD background and enables the liver repopulation with human hepatocytes[130,133]. Animals used for these studies were 5-6 weeks old and were maintained on regular protein intake diet (PicoLab High Energy Mouse Diet, 19% protein). Animals were pre-treated with an adenovirus vector expressing uPA (Yecuris Corp.) approximately 24 h before transplantation. Hepatocytes were transplanted directly into the spleen. In both studies, mice received around 1 million viable cells suspended in 200 µL plasmalyte. Analgesic treatment was administered prior and 24 h post transplantation. Blood samples were collected starting from approximately week 5 post transplantation. Human albumin was quantified as indicated in the respective section above.

3.10 AMMONIA ASSAY AND UREAGENESIS

In vivo ammonia measurements were performed when animals reached the desired repopulation levels (higher than 80% in **PAPER IV**, higher than 80% or full repopulation in **PAPER V**). For basal ammonia measurements, 5 µL blood were diluted in 20 µL water, and samples were consequently analysed with Arkray Pocket Chem (Arkray). Ammonia challenge was performed with injection of 4 mmol/kg of body weight $^{15}NH_4Cl$ and measured 30 min later. In **PAPER IV**, urea was additionally evaluated using blood samples from the ammonia challenge and analysed as described by Allegri et al.[256].

3.11 URINARY OROTIC ACID, ENZYME ACTIVITIES AND AMINO ACIDS

In vivo phenotype characterization was performed in **PAPERS IV** and **V** measuring OTC and CPS1 enzyme activities, as well as urinary orotic acid and amino acids. OTC and CPS1 enzyme activities were quantified following published protocols.[257] Amino acids analysis was performed in the clinical laboratory at Karolinska Institutet using mass spectrometry. For detailed protocols on the above, as well as on urinary orotic acid quantification, readers are redirected to **PAPERS IV** and **V**.

3.12 STATISTICAL ANALYSIS

Statistical analysis was performed using Graph Pad Prism 6.0 software. Ordinary ANOVA and Tukey multiple comparison tests or Kruskal-Wallis ANOVA and Dunn's multiple comparison tests were applied to compare means of three experimental groups when normally distributed or not, respectively. In order to compare means of two groups of normally distributed data set or not, a two-tailed, unpaired t-test or Mann-Whitney U test were applied, respectively. The level of significance was set at p-value <0.05 (* $p<0.05$; ** $p<0.01$; ***$p<0.001$; ****$p<0.0001$). Statistical analysis of targeted amplicon deep sequencing data, as well as information on bioinformatic pipelines used for CIRCLE-seq are described in **PAPER V**.

4 RESULTS

4.1 PAPER I

Title: Applying hydrodynamic pressure to efficiently generate induced pluripotent stem cells via reprogramming of centenarian skin fibroblasts.

4.1.1 Study overview

Dermal fibroblasts from young and centenarian individuals were obtained and reprogrammed into iPSC through an optimized reprogramming protocol without genome integration and under feeder-free conditions. Pluripotency of generated iPSC clones was confirmed with various methods. A graphical abstract of the study is presented in Figure 7.

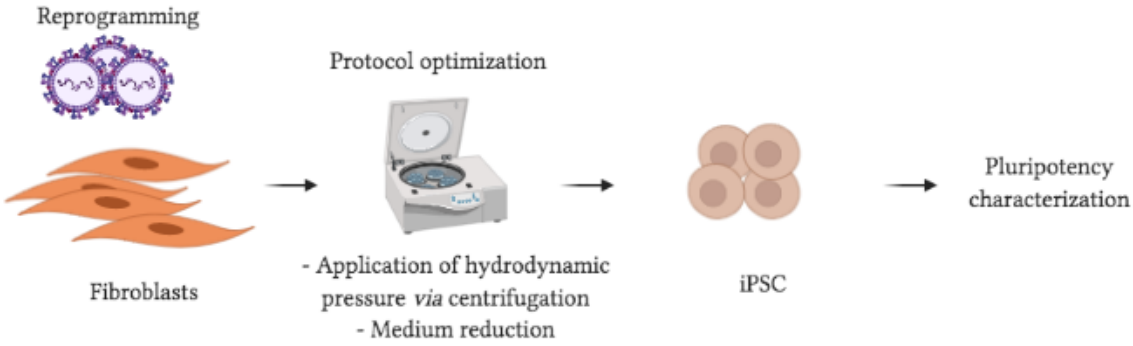

Figure 7: Graphical abstract of PAPER I. Created with Biorender.com.

4.1.2 Results

Reprograming of somatic cells from old individuals is frequently considered challenging when successful at all; therefore, we revised currently used protocols aiming to increase the reprogramming efficiency. Noteworthy, the dermal fibroblasts utilized in this study were derived from centenarian individuals (105-107 years old), rather than patients with liver genetic defects that are the focus of this thesis. My involvement in this study was mainly educational, as a mean to gain adequate experience and knowledge on somatic cell reprogramming and iPSC culture, essential for **PAPERS II** and **III**.

To summarize the results, reprogramming experiments were performed following manufacturer's instructions and introducing few modifications. Our revised protocol includes hydrodynamic pressure by centrifugation, as well as reduction of medium volume used during the viral exposure. These modifications enhanced the transduction efficiency of fibroblasts derived both from centenarian and young donors, used as study controls. Specifically, transduction efficiency of cells from young donors (nHF, aHF) increased from 19.5 ± 5.2% and 11.7 ± 1.7% to 49.3 ± 4.9% and 31.0 ± 2.8%, respectively, while the efficiency of cells from centenarian donors (cHF1, cHF2) improved from 5.3± 1.5% and 7.5 ± 1.9% to 13.3 ± 2.1% and 17.3± 2.2%, respectively, as shown in Figure 8.

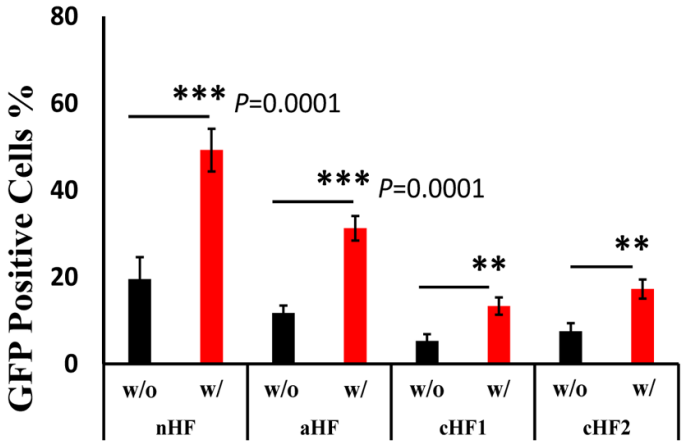

Figure 8: Evaluation of transduction efficiency. Comparison of GFP positive fibroblasts derived from young donor controls (nHF and aHF) and centenarian donors (cHF1 and cHF2), with (w/) or without (w/o) centrifugation. Means were compared applying paired t-test. Biological replicates n=3.

Five selected iPSC clones were characterized for pluripotency based on various methods. First, the cells were positively stained for alkaline phosphatase, a marker for stem cells (Figure 9). Another method to test pluripotency is PluriTest which examines the gene expression level on a global scale and compares the transcriptomic profiles of newly generated iPSC clones with those of already known and proven pluripotent or differentiated, somatic cells. PluriTest estimates pluripotency and novelty scores, with the first showing profiles associated with pluripotency while the latter indicating features of gene expression consistent with differentiation.[252,253] All iPSC clones subjected to the assay had high pluripotency and low novelty scores when compared to parental, somatic cells and other negative internal controls. Furthermore, whole transcriptomic analysis was validated with qRT-PCR showing high gene expression of *NANOG*, *SOX2* and *OCT4*. Moreover, pluripotency was investigated on the protein level with flow cytometry and immunocytochemistry. Results indicated high positivity for the nuclear and surface pluripotent markers NANOG, SOX2, SSEA3 and TRA1-81, while iPSC were negative for SSEA1, a maker associated with differentiation and loss of pluripotency (data provided in **PAPER I**).

In conclusion, the study presents evidence that minor modifications, such as a centrifugation step and reduction of medium volume significantly increases the reprogramming efficiency, even in centenarian cells, which are considered difficult substrate for reprogramming.

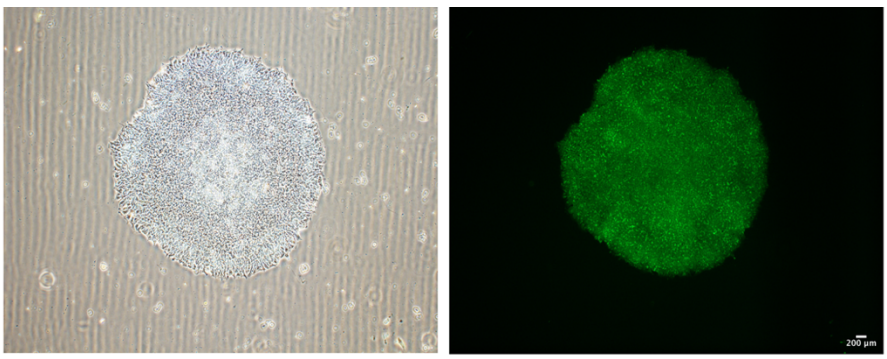

*Figure 9: Morphology of an iPSC colony derived from a centenarian donor (**left**). Representative iPSC colony derived from fibroblasts from a centenarian donor stained for alkaline phosphatase (**right**).*

4.2 PAPER II

Title: Guide to the assessment of mature liver gene expression in stem cell-derived hepatocytes.

4.2.1 Study overview

Gene expression of more than 60 genes was analyzed in 17 fetal and 25 mature (postnatal) human liver tissues. We propose that the generated dataset provides a simple and accurate assessment tool for hepatic maturity of stem cell-derived HLC, which can serve as a guide for investigators to evaluate and improve hepatic differentiation protocols. Graphical abstract of the study is presented in Figure 10.

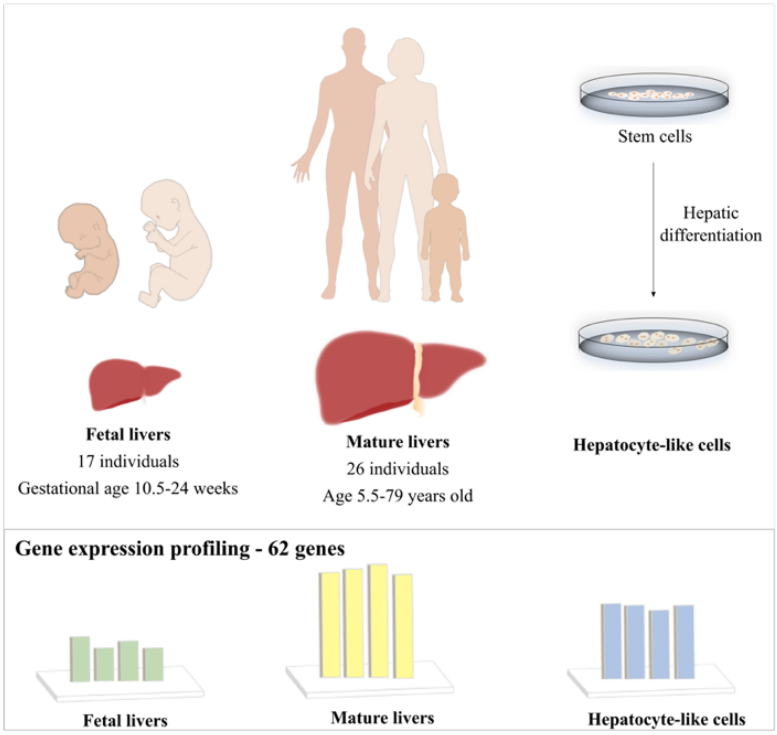

Figure 10: Graphical abstract of PAPER II.

4.2.2 Results

Liver fibroblasts were reprogrammed into iPSC and the selected clones were characterized, as previously described in **PAPER I** (data not shown). HLC organoids (HLC ORG) were generated through a four-step protocol; endoderm induction, hepatoblast formation, organoid development and hepatic differentiation. Depiction of the protocol applied, as well as representative images from the last stage of differentiation are presented in Figure 11. Several organoid bodies attached to the bottom of the culture plate, facilitating the visualization of HLC morphology (Figure 11, right). Gene expression in HLC generated in our laboratory, as well as HLC from three commercial sources were analyzed and compared to the respective levels in fetal and mature (postnatal) liver tissues. It needs to be stressed that the main aim of this paper was not to describe an optimal differentiation procedure or compare our HLC with the commercial cells, but rather to provide a quick and accurate data set, easily applicable in any laboratory for the assessment and improvement of differentiation procedures.

The data set included a total number of 62 genes categorized as liver-specific proteins, Phase I and II, nuclear factors, transporters, multidrug resistance and cytoskeletal proteins, as well as pluripotency and early differentiation genes. Cells generated in our laboratory were denoted ORG HLC, while those from pharmaceutical companies Commercial HLC A, B and C.

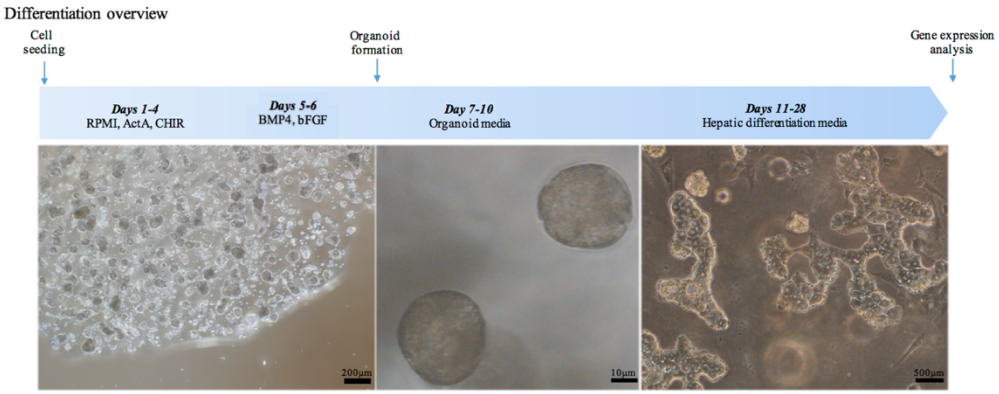

Figure 11: Schematic representation of hepatic differentiation protocol used in the study, along with representative images at the end of the experiment.

Common indicators of hepatic maturity are *ALB* and *AFP*. We observed no difference in *ALB* expression between fetal and mature liver tissues, with ORG HLC and two commercial HLC being in the range of mature liver cells. On the contrary, as expected, there was a large difference in *AFP* expression between fetal and postnatal tissues. All HLC failed to down-regulate the levels to mature tissues, indicating residual fetal characteristics. Furthermore, fetal and mature livers displayed similar expression of genes encoding hepato-specific proteins such as *A1AT*, glutamate-ammonia ligase (*GLUL*), *CPS1*, *PAH* and *FAH*. HLC generated in our laboratory were in the range of mature levels for these genes, whereas lower levels were observed in the commercial HLC. Finally, regarding *OTC* expression, only HLC A and HLC C reached the lowest range found in mature samples.

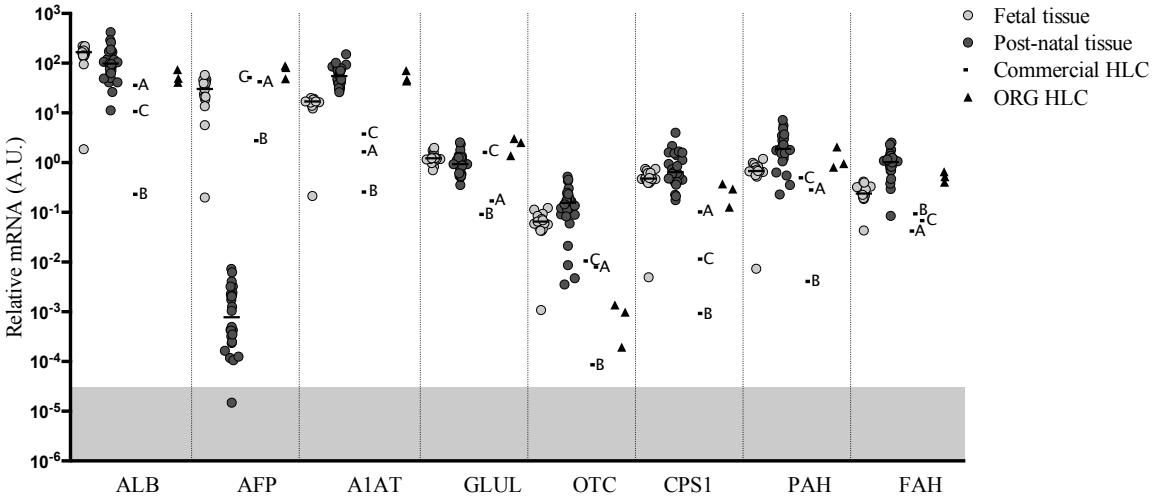

Figure 12: Gene expression levels of liver-specific plasma proteins and metabolic enzymes normalized to endogenous control Peptidylprolyl Isomerase A (PPIA) in fetal and post-natal liver tissues, as well as commercial hepatocyte-llike cells (HLC) (denoted A, B and C) and organoid HLC generated in our laboratory (denoted ORG HLC). Horizontal bars represent median values. Gray-highlighted area indicates unreliable levels of expression.

Furthermore, we performed analysis of hepatic enzymes involved in the metabolism of endogenous and exogenous compounds, such as cytochrome P450 genes (Figure 13). Briefly, the results showed that all HLC failed to reach mature levels of CYP2D6. Furthermore, CYP3A7 and CYP3A4 are considered important CYP genes, being often referred as fetal and adult CYP3A genes, respectively. Two commercial HLC and ORG HLC displayed expression of CYP3A4 at the lower levels of mature hepatic tissue, while still expressing the fetal CYP. Genes involved in cholesterol metabolism and bile acid synthesis were additionally investigated. Specifically, all HLC except commercial source B reached mature levels of CYP7A1, while only HLC ORG displayed equivalent expression of CYP7B1 to mature liver samples. Readers are referred to **PAPER II** for results on other genes investigated.

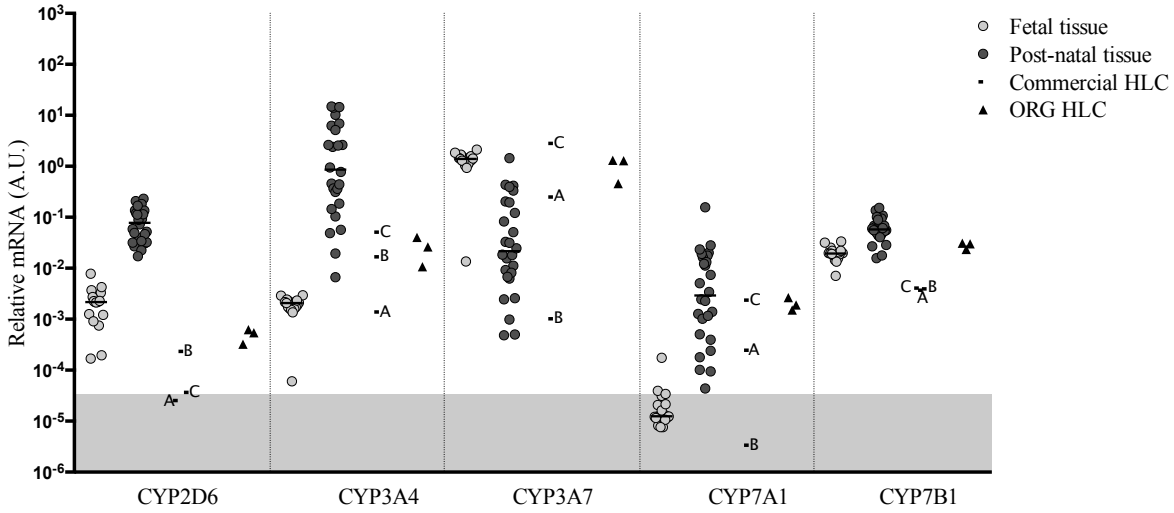

Figure 13: Gene expression levels of cytochrome P450 genes normalized to endogenous control Peptidylprolyl Isomerase A (PPIA) in fetal and post-natal liver tissues, as well as commercial hepatocyte-like cells (HLC) (denoted A, B and C) and organoid HLC generated in our laboratory (denoted ORG HLC). Horizontal bars represent median values. Gray-highlighted area indicates unreliable levels of expression.

4.3 PAPER III

Title: Gene editing correction of a urea cycle defect in organoid stem cell derived hepatocytes.

4.3.1 Study overview

Liver fibroblasts from an OTCD patient were isolated and reprogrammed into iPSC. The cells were consequently genetically corrected, differentiated into organoid HLC and phenotypically characterized *in vitro*. A graphical abstract of the study is presented in Figure 14.

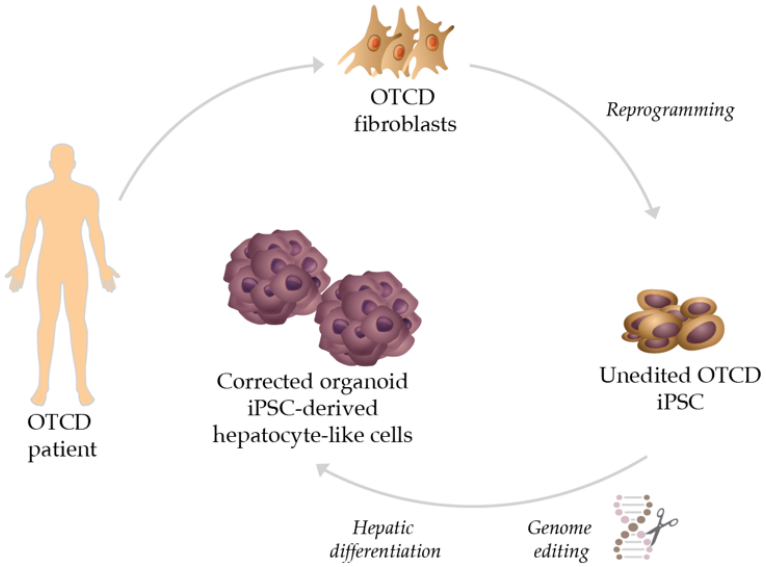

Figure 14: Graphical abstract of PAPER III.

4.3.2 Results

The disease-causing variant in the OTCD patient was investigated by applying long-range PCR for the amplification of whole *OTC* gene, and subsequent amplicon sequencing through targeted next generation sequencing. When sequencing reads were aligned to a reference *OTC* gene (NCBI), one previously reported pathogenic variant was identified (c.G>A, rs66656800).[254] This causes the generation of three distinct *OTC* mRNAs in the affected patient; skipping of exon 4 (r.299_386del), elongation of exon 4 with the first 4 bp of intron 4 and spliced by a cryptic splice site in intron 4 (r.386_387ins386+1_386+4), and finally the full length of transcript naturally spliced containing exon 4 and harbouring the mutation (r. 386g>a), as illustrated in Figure 15.

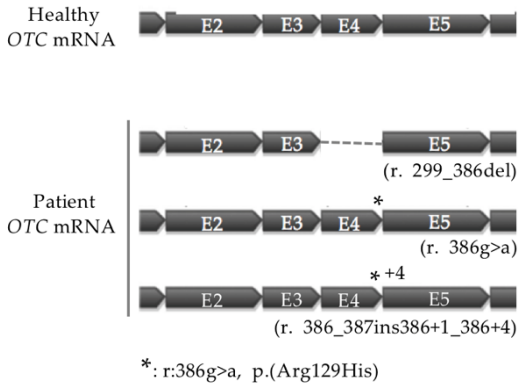

Figure 15: Representation of OTC transcripts in healthy (OTC proficient) and OTCD patient hepatocytes.

Six iPSC clones were generated and characterized (data shown in **PAPER III**), one of which was chosen for further studies because of its higher pluripotency and differentiation capacity into the endoderm lineage. When CRISPR/Cas9 technology was applied, the editing efficiency was estimated to be approximately 10%, based on restriction enzyme digestion assay (Figure 16a). Next, the mixed population of unedited and edited iPSC were clonally expanded, and ultimately a genetically corrected clone was isolated, as shown in Figure 16b. Successful correction was confirmed by sequencing of the genomic *locus* of interest (Figure 16c), as well as with investigation of the *OTC* transcript (Figure 16d). Specifically, there were two bands evident when *OTC* transcript was amplified in OTCD primary hepatocytes and unedited iPSC-HLC, while only a wild-type transcript was observed in edited iPSC-HLC and OTC proficient primary hepatocytes (Figure 17d).

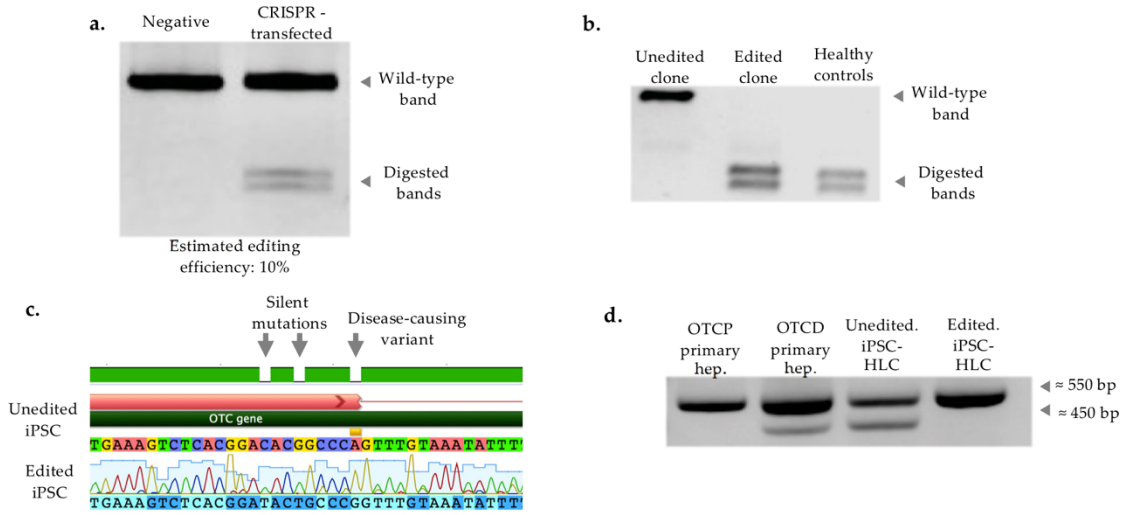

Figure 16: Validation of genome editing. ***(a)*** *Assessment of editing efficiency. Evaluation of editing efficiency was conducted with restriction enzyme assay and estimated based and intensities of wild-type and cleaved bands. Negative: untransfected cells. CRISPR-transfected: cells transfected with Cas9 and gRNA.* ***(b)*** *Restriction enzyme assay and clone screening. The successful correction in the screened clones was indicated by the cleaved bands after the restriction enzyme assay, similar as observed in healthy control, while unedited clone displayed only a wild-type amplicon.* ***(c)*** *Validation of successful genome correction through sequencing of genomic DNA in unedited and edited iPSC clones.* ***(d)*** *Investigation of OTC transcript. Amplification of OTC transcript was performed in OTC proficient (OTCP) and OTCD primary hepatocytes, as well as in unedited and edited iPSC hepatocyte-like cells (iPSC-HLC).*

Thereafter, unedited and edited iPSC were differentiated into HLC through organoid formation. Gene expression profiling was evaluated at different stages of differentiation in both unedited and edited iPSC-HLC. In general, levels of gene expression were changed throughout different differentiation stages, suggesting the transition from stem cells to endoderm, hepatoblast and finally HLC. Remarkably, there were no obvious differences between the genetically engineered and the parental cells for most of the genes (data shown in **PAPER III**).

Finally, phenotypic characterization, *in vitro,* was also performed (Figure 17). Albumin was measured on days 20 and 26 of hepatic differentiation protocol, and there were no significant differences between unedited and edited HLC (Figure 17a). Furthermore, we measured urea production since it would be anticipated that successful *OTC* gene correction would restore urea cycle activity. Indeed, we observed significantly different levels in the ratios of labelled/unlabelled urea between unedited and edited HLC, both after 20 and 26 days of *in vitro* differentiation (Figure 17b).

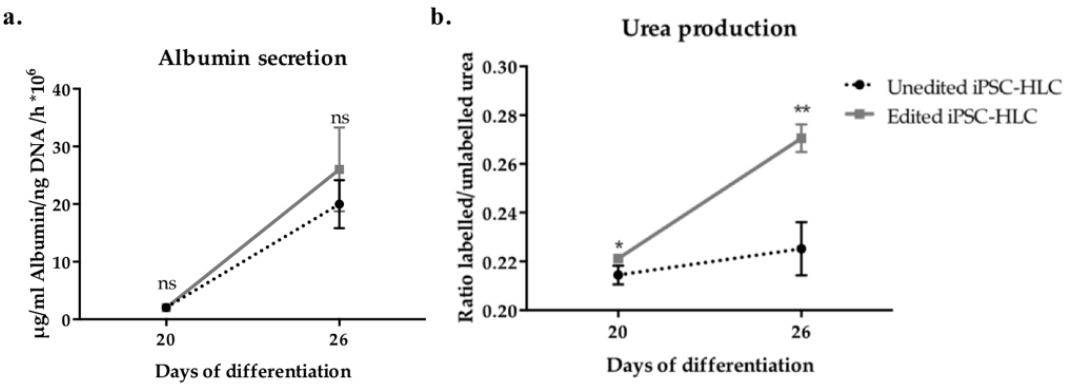

Figure 17: In vitro phenotypic characterization. ***(a)*** *Albumin secretion by unedited and edited iPSC-HLC was measured on days 20 and 26 of hepatic differentiation. Biological replicates n =3.* ***(b)*** *Incorporation of ^{15}N into urea which is an indicator of urea produced through the urea cycle. Labelled and unlabeled urea were quantified with mass-spectrometry on days 20 and 26 of hepatic differentiation. Biological replicates n=6. Averages and errors are shown as means and standard deviation. Mann Whitney U test was used for statistical analysis. Ns: not significant; *: p<0.05; **: p<0.01.*

Finally, a concern when applying genome editing technologies are unspecific and unintended genetic alterations that might occur. To this end, we performed whole genome sequencing on unedited and edited iPSC cells. None of the differences identified between the clones were a result of CRISPR application, indicating that no off-target mutagenesis occurred (data shown in **PAPER III**).

4.4 PAPER IV

Title: A liver-humanized mouse model of carbamoyl phosphate synthetase 1 deficiency.

4.4.1 Study overview

Primary hepatocytes from CPS1 proficient (CPS1P) donors and a CPS1D patient were transplanted into FRGN mice. Phenotypic characterization was carried out on highly repopulated, liver-humanized animals to determine if they recapitulate clinical manifestations observed in affected CPS1D patients, and subsequently conclude if the mouse could be used as a mouse model of the disease. A graphical abstract of the study is presented in Figure 18.

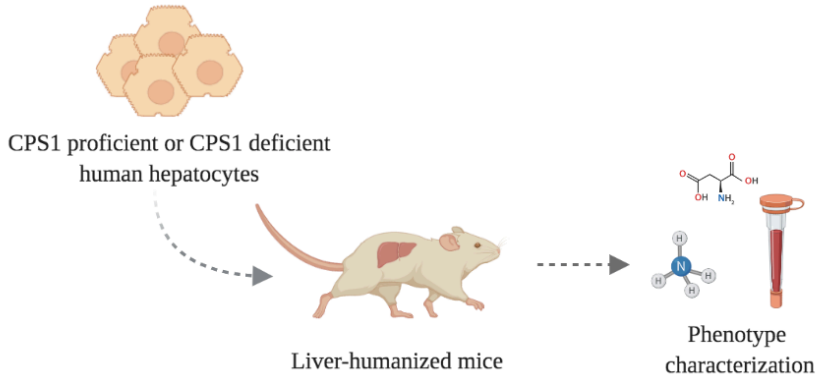

Figure 18: Graphical abstract of PAPER IV. Created with Biorender.com.

4.4.2 Results

FRGN mice were transplanted with primary human hepatocytes either from CPS1P or CPS1D donors, and the levels of liver humanization were estimated based on human albumin in the mouse sera. It has been estimated from previous studies that every 1 mg/ml of human albumin corresponds to approximately 20% repopulation of the liver.[130,133] All animals with 80% or higher liver humanization (> 4 mg/ml human albumin) were included in the study, and there was no significant difference in albumin levels between the groups (Figure 19).

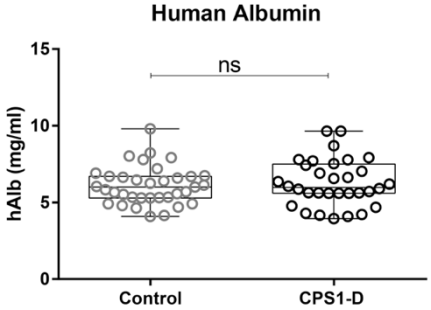

Figure 19: Levels of circulating human albumin in mice liver-repopulated with control (CPS1 proficient) or CPS1 deficient (CPS1D) hepatocytes. Averages and errors are presented as medians and interquartile ranges. Mann-Whitney U test was used to analyze the data sets. Ns: not significant.

Hyperammonemia is hallmark of CPS1D; therefore, blood ammonia levels were among the markers evaluated. While ammonia levels were numerically higher in the group that received CPS1D cells, there was no statistically significant difference between CPS1D and controls (182 µM and 148 µM, respectively) (Figure 20a). However, when the mice were challenged with the administration of exogenous $^{15}NH_4Cl$, the CPS1D group metabolized ammonia at significantly lower rates than the controls (Figure 20b). Furthermore, we sought to quantify the enrichment of ^{15}N into urea following the administration of $^{15}NH_4Cl$. As shown in Figure 20c, there was a significant difference in ^{15}N incorporation into urea between the two experimental groups (Control: 11%, CPS1D: 7%).

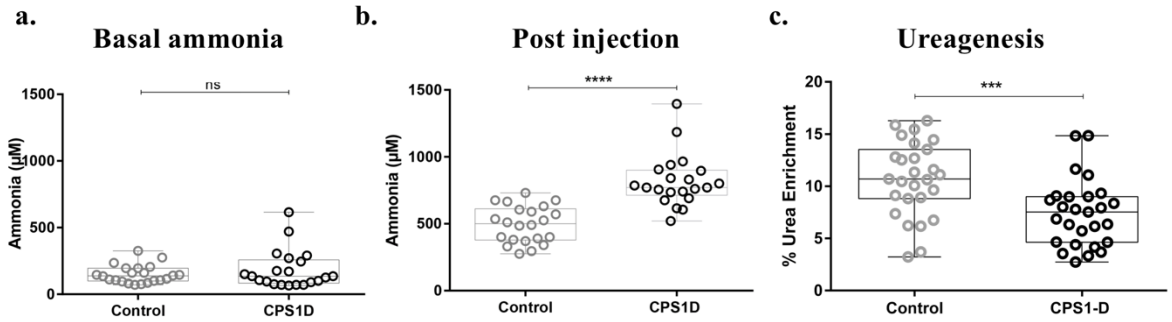

Figure 20: Ammonia levels and isotopic enrichment of ^{15}N into urea in liver-humanized mice transplanted with control (CPS1 proficient) or CPS1 deficient (CPS1D) hepatocytes. When mice highly humanized, ammonia levels were measured before (Basal ammonia) ***(a)****, as well as 30 min post the administration of exogenous $^{15}NH_4Cl$ (Post injection)* ***(b)****. **(c)** The incorporation of ^{15}N into urea was measured 30 min post the administration of exogenous $^{15}NH_4Cl$. Averages and errors are presented as medians and interquartile ranges. Mann-Whitney U tests were used to analyze the data sets. Ns: not significant; *** p< 0.001; **** p<0.0001.*

In addition to ammonia and urea which are the substrate and end product of the cycle, amino acids derangements are also observed in CPS1D patients. Therefore, we investigated the amino acid profile, and found that blood glutamine and glutamate were significantly different between the mice that received defective for the *CPS1* gene cells and those that were injected with CPS1P hepatocytes (Figure 21a-b). In addition, CPS1 enzyme activity in the liver of CPS1D mice was by 80% lower than the control group. We also quantified OTC enzyme activity in the same samples with no difference being observed (Figure 21b). Finally, we were interested in exploring if the expression of liver mature genes was altered in CPS1D mice, and found no differences in the gene expression levels of the genes investigated (data shown in **PAPER IV**).

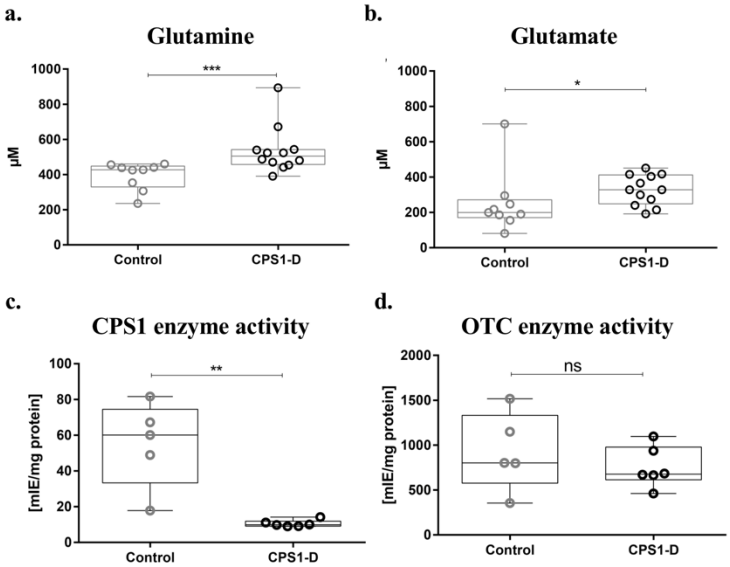

Figure 21: Amino acids and enzyme activities in liver-humanized mice transplanted with control (CPS1 proficient) or CPS1 deficient (CPS1D) hepatocytes. Glutamine ***(a)*** *and glutamate* ***(b)*** *were measured in the blood of repopulated animals. CPS1* ***(c)*** *and OTC* ***(d)*** *enzyme activities were quantified in the livers. Averages and errors are presented as medians and interquartile ranges. Mann-Whitney U tests were used to analyze the data sets. Ns: not significant; * p<0.05; **p<0.01; *** p< 0.001.*

4.5 PAPER V

Title: Correction of a urea cycle defect after *ex vivo* gene editing of human hepatocytes.

4.5.1 Study overview

Hepatocytes derived from an OTCD patient were genetically edited *ex vivo* through a CRISPR-mediated dual gRNA approach and phenotypic restoration of urea cycle activity was demonstrated *in vitro*, as well as *in vivo* in liver-humanized mice. In addition, safety of *ex vivo* correction of primary human hepatocytes was investigated through gene expression and CIRCLE-seq-based deep sequencing analysis. A graphical abstract of the study is presented in Figure 22.

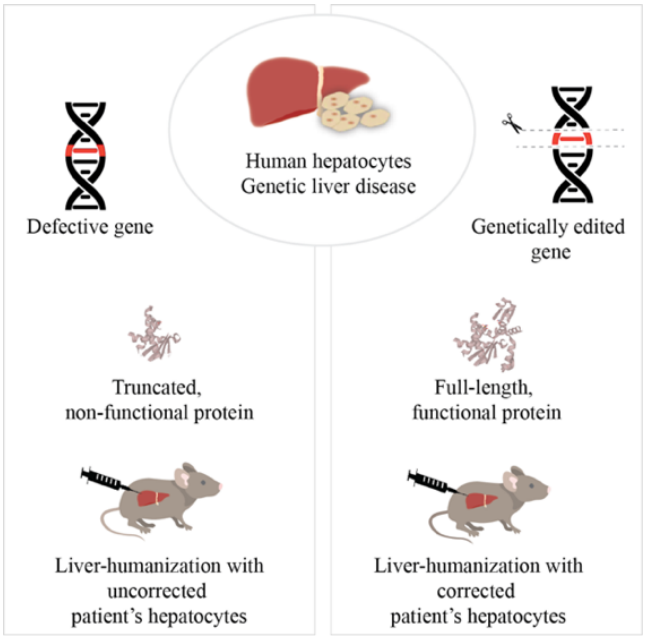

Figure 22: Graphical abstract of PAPER V.

4.5.2 Results

First, we sought to identify the mutation that caused the OTCD in this particular patient through *OTC* transcript amplification. The investigation revealed the presence of two transcripts of different lengths; a wild-type and an elongated transcript by approximately 130 bp (Figure 23). Sequencing of *OTC* mRNA and the suspected DNA region containing the mutation showed that a point mutation in intron 5 (c.540+265G>A) generated a splice acceptor site incorporating an intronic region in the *OTC* mRNA and introducing a premature stop codon shortly after exon 5 (p. Q180_E181insCHI*), as graphically represented in Figure 24.

Since no other mutations were observed in the coding region, we reasoned that deletion of the intronic sequence containing the mutation would most probably restore normal splicing and correct the deficiency. Therefore, we applied a dual gRNA approach on primary hepatocytes with reported editing efficiency higher than 60%, estimated based on amplicon intensities of wild type and cleaved bands, and corrected for their lengths (Figure 25). Correction of the *OTC* gene would be expected to restore urea cycle and increase the amount of urea produced by the cells. To this end, unedited and edited hepatocytes were incubated

with $^{15}NH_4Cl$ and ratios of labelled/total urea were quantified on days 2, 4 and 6 post electroporation through mass spectrometry. We observed a significant difference between unedited and edited primary hepatocytes in their capacity to incorporate ^{15}N into urea signifying the restoration of urea cycle (Figure 26).

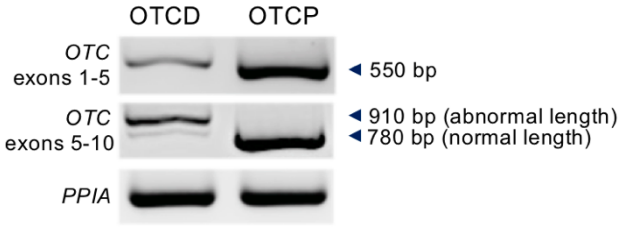

Figure 23: OTC transcript amplification in hepatocytes from OTC deficient (OTCD) patient and OTC proficient (OTCP) donor. The length of each transcript is indicated with arrows. Peptidylprolyl Isomerase A (PPIA) transcript served as endogenous control.

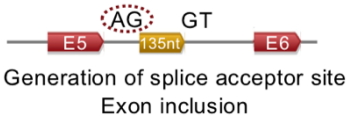

Figure 24: Genomic structure of exons 5 and 6 (red boxes) of the OTC gene and the intronic region introduced in the OTC transcript (yellow box). Dinucleotides of the generated splice acceptor (AG) and the naturally existing donor splice site (GT) are shown.

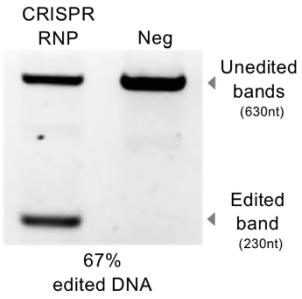

Figure 25: Quantification of editing efficiency. PCR amplification of the target genomic region in edited (CRISPR riponucleoprotein; RNP) and unedited (Negative; Neg) primary hepatocytes. Higher and lower bands indicate unedited and edited DNA, respectively. Length of each band is indicated with arrows. Editing efficiency was quantified based on band intensities and corrected for their length.

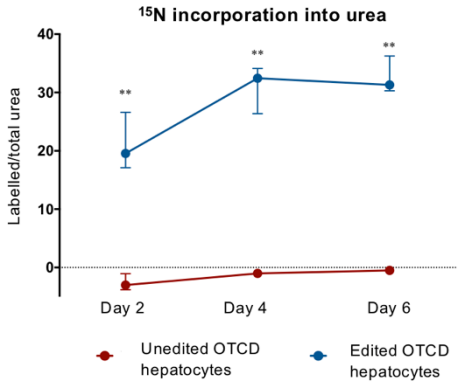

*Figure 26: Phenotypic characterization in vitro. Unedited and edited primary hepatocytes were incubated with $^{15}NH_4Cl$ on days 2, 4 and 6 post electroporation and ratios labelled/unlabeled urea were measured with mass-spectrometry. Values and errors represent medians and interquartile ranges. Biological replicates n=6. Mann-Whitney U test was used to analyse the datasets. **p<0.01.*

Given the encouraging results from the *in vitro* phenotype characterization, we proceeded with the transplantation and liver humanization of FRGN mice with unedited OTCD, edited OTCD or OTC proficient (OTCP) hepatocytes. When mice were highly humanized, they were subjected to a series of assessments, including ammonia and urinary orotic acid, as well as OTC and CPS1 enzymes activities (Figure 27). Briefly, basal ammonia in mice that received edited OTCD cells was significantly lower than in those transplanted with unedited OTCD, and at similar levels to the OTCP group. Additionally, when mice were subjected to ammonia challenge by the injection of exogenous NH_4Cl, edited mice could clear ammonia faster than the unedited counterparts, however they did not reach the levels observed in the OTCP controls. In addition to hyperammonemia, another hallmark of the disease is the elevated urinary orotic acid which is a characteristic surrogate marker of OTCD because the

accumulated carbamoyl phosphate, the substrate for OTC, enters the pyrimidine nucleotide pathway and produces orotic acid. Edited mice displayed lower urinary orotic acid than the unedited group, with half of the mice being in the range of the OTCP group. Furthermore, values of OTC enzyme activities in edited mice were normalized as we observed no statistical difference between the edited and control groups. In addition, we measured the activity of another enzyme of the urea cycle, CPS1, and as expected there were no differences between any groups (Figure 27). Expression of OTC protein in mice that received edited OTCD cells was confirmed with immunohistochemistry. On the contrary, liver of mice repopulated with unedited OTCD hepatocytes showed almost no antibody staining against the protein (data shown in **PAPER V**).

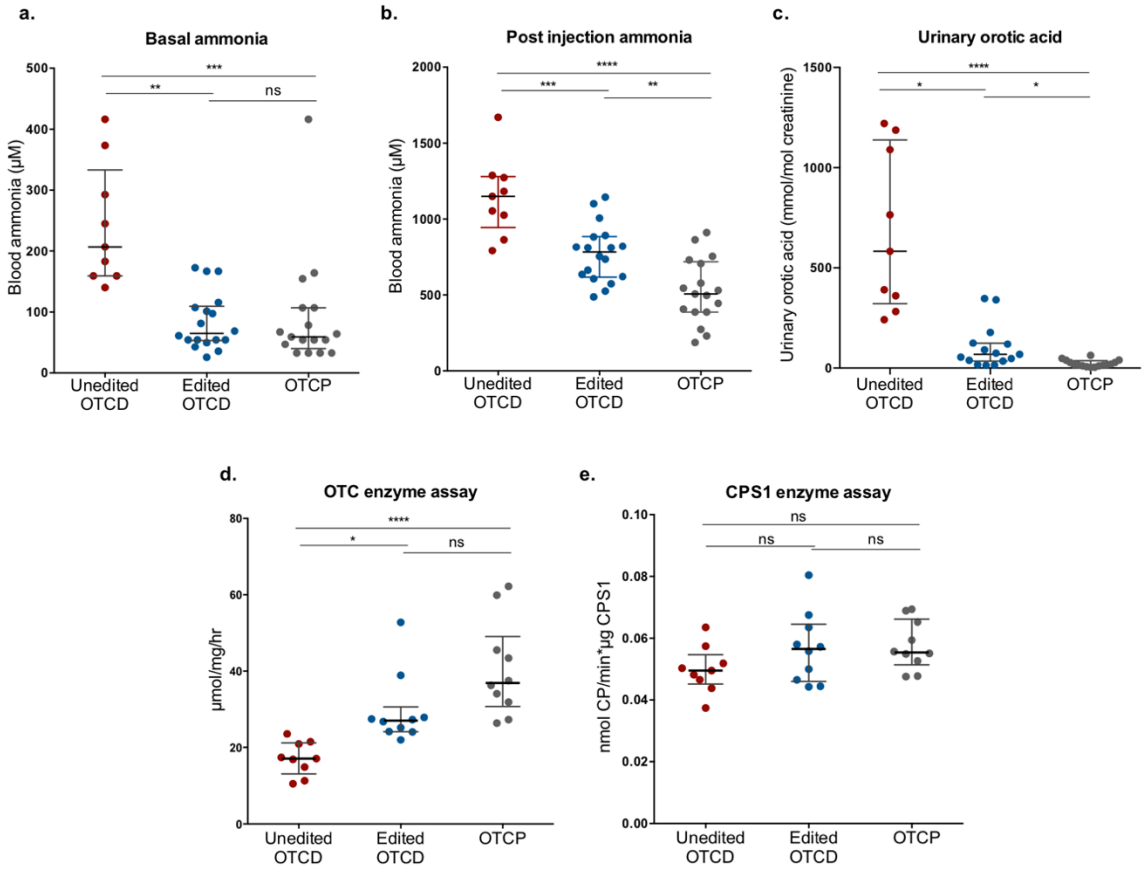

Figure 27: In vivo phenotype characterization of liver-humanized mice with unedited or edited OTC deficient (OTCD) or OTC proficient (OTCP) hepatocytes. ***(a)*** *Basal blood ammonia in humanized mice. Unedited OTCD mice n=9; Edited OTCD mice n=18; OTCP mice n=17.* ***(b)*** *Blood ammonia levels 30 min post administration of NH^4Cl (ammonia challenge). Unedited OTCD mice n=9; Edited OTCD mice n=18; OTCP mice n=17.* ***(c)*** *Urinary orotic acid in humanized mice normalized to urinary creatinine. Unedited OTCD mice n=9; Edited OTCD mice n=15; OTCP mice n=16.* ***(d)*** *OTC enzyme activity in humanized mouse livers. Unedited OTCD mice n=9; Edited OTCD mice n=10; OTCP mice n=10.* ***(e)*** *CPS1 enzyme activity in humanized mouse livers. Unedited OTCD mice n=9; Edited OTCD mice n=10; OTCP mice n=10. Averages and errors are shown as medians and interquartile range. Kruskal-Wallis ANOVA and Dunn's multiple comparison tests were used to analyze datasets not normally distributed (basal ammonia, urinary orotic acid, and OTC enzyme activity), while ordinary ANOVA and Tukey multiple comparison tests for datasets normally distributed (post-injection ammonia and CPS1 enzyme activity).*

Once efficient gene correction established, we investigated the safety of such application. Gene expression profiling was conducted assessing more than 30 genes in livers humanized either with unedited or edited cells, revealing similar level of expression (data shown in **PAPER V**). Moreover, genome-wide potential off-target mutagenesis was evaluated

applying CIRCLE-seq, which is an unbiased method relying on the actual genome, instead of reference sequences.[258] Deep sequencing of more than 70 *loci* indicated no unspecific genomic alterations, pointing out that the *ex vivo* correction of OTCD primary hepatocytes was safe (data shown in **PAPER V**).

5 DISCUSSION AND FUTURE DIRECTIVES

IEM are a group of more than 500 heterogeneous disorders resulting from defects in metabolic pathways. While these diseases are individually rare, they are characterized by high cumulative prevalence. Specifically, the incidence of all-cause IEM is estimated to be 60 per 100,000 live births which translates to more than 70,000 new IEM cases per year globally. Additionally, these conditions are characterized by high fatality rates, resulting in more than 23,000 deaths annually which accounts for 0.4% of all child deaths worldwide.[259] While our understanding of IEM has remarkably broadened over the last two decades, there is a significant gap regarding treatments and disease models that could be used to study these pathologies. Herein, we sought to investigate potential *in vitro* and *in vivo* models of UCD, which could certainly be extended to other liver genetic diseases, as well as to explore the possibilities that genome engineering could offer both for disease modelling and therapeutic alternatives.

Harnessing the potentials of iPSC might assist in achieving the aforementioned objectives. However, their generation through somatic cell reprogramming is frequently challenging or inefficient. In **PAPER I,** we managed to improve the reprogramming protocol with the introduction of a centrifugation step and reduction of medium volume during viral exposure which increased the transduction efficiency in cells both from exceedingly old individuals and young donors. The obtained clones met all the criteria of full reprogramming, including expression of pluripotency markers, self-renewal capacity and lack of large chromosomal abnormalities. Conclusively, **PAPER I** provides evidence that minor modifications in the reprogramming procedure can improve the reprogramming efficiency, and perhaps increase the convenience of using iPSC in regenerative medicine or disease modelling. The methodology developed here was applied in other studies of the thesis (**PAPERS II, III** and **V**).

A large body of literature exists investigating potential ways to generate stem cell-derived HLC. The reason why these cells are named "hepatocyte-like cells" (HLC) in this thesis and its constituent papers is that they acquire some hepatic features when subjected to differentiation protocols, but many of these characteristics are similar to those present in fetal hepatocytes, rather than adult cells. Unfortunately, researchers often over-interpret their data with most papers inflating their titles in an attempt to convince the reader that "mature" hepatocytes were produced, even though in many publications no actual human hepatocytes or liver tissues are included in the analysis. The problem arises in part from the lack of access to reliable human liver tissue or cell samples in most laboratories. In addition, the intraindividual variability of hepatic functions demands the use of hepatocytes or liver tissues from multiple donors. To this end, in **PAPER II** we sought to generate a simple and accurate assessment tool for the evaluation of hepatic differentiation protocols based on qPCR. While we acknowledge that emerging technologies in the field of single cell transcriptomics might reveal more information than testing individual genes with qPCR, these techniques are characterized by high cost, complexity and demand high bioinformatic skills. Other methods for protein instead of RNA quantification (*e.g.* ELISA, mass spectrometry, immunocytochemistry), or techniques that measure phase I and II metabolic activity could have been also applied. However, the multiplexity of these methods makes them not amenable to standardization and comparison between laboratories. Therefore, we aimed to generate qPCR-based gene expression profiles of fetal and mature (post-natal) liver tissues

using commercially available reagents so that researchers could easily graph the data from their own HLC and compare them with actual hepatic profiles. The large number (17 fetal and 25 mature) of human liver tissues included in the study provides a robust data set displaying the large variations in gene expression observed between individuals and significantly improves the value of this approach to evaluate the state of differentiation of the cells. Finally, it needs to be stressed that the aim of **PAPER II** was to provide an assessment tool for hepatic differentiation, rather than to describe an optimal differentiation protocol.

To demonstrate the applicability of the proposed tool, iPSC-derived HLC generated in our laboratory, as well as HLC commercially procured were incorporated in the mini-array. We note that HLC in **PAPER II** had expression levels within the fetal range for some genes, and within the mature range for others. Commonly used markers for HLC characterization are *CYP3A4* and *CYP3A7* which are strongly age-dependent. CYP3A7 is an enzyme required for the fetal growth and development, and the metabolism of endogenous compounds. Expression of *CYP3A7* decreases during the first postnatal years to low adult levels with a concomitant increase in *CYP3A4* expression that may reach mature levels in the adolescence. Our cells displayed fetal levels of *CYP3A7* and overlapping levels with the lowest values of *CYP3A4* suggesting an intermediated phase between fetal and mature status. Data on *ALB* and *AFP* expression, which are mature and fetal markers, respectively, followed the same pattern. Surprisingly, transcription factors that are known to be crucial for the regulation of mature liver genes (*e.g. HNF1*, *HNF3*, *HNF4*, *HNF6*, *LXRa*, *FXR*) were found to be expressed at or near normal mature levels. Therefore, we reasoned that their expression is required but not sufficient for complete maturation of iPSC-HLC. Another important observation of the study is the considerable variability between donors, particularly for the CYP genes, reinforcing the need for inclusion of hepatocytes from multiple donors when efficacy of differentiation protocols is evaluated.

In spite of more than 40 hepatic differentiation experiments conducted for **PAPERS II** and **III**, each lasting almost a month, we did not generally achieve complete maturation of HLC. Substantial improvements were noticed with optimizations of coating material, cell seeding density, endoderm induction protocols etc. (**PAPER III).** Additionally, it is generally accepted that different clones might exhibit diverse differentiation propensity into specific lineages. Indeed, experiments in **PAPER III** strikingly indicated this fact when several clones were subjected to endoderm induction displaying different capacity to transition to endoderm cells, even though they were obtained from the same donor, generated during the same reprogramming experiment and simultaneously expanded. Finally, significant improvement of differentiation was achieved through the formation of three-dimensional structures (organoids), as has been reported by others as well.[50,52,104]

Even though organoid formation improved our differentiation protocols, hepatic maturation remained incomplete and was further illustrated in **PAPER III**. In this study, we achieved the generation of iPSC from an OTCD individual and genetic correction of the pathogenic variant that accounted for the UCD. While we demonstrated the correction on molecular level and showed a phenotypic difference between unedited and edited HLC through assessment of ^{15}N incorporation into urea, urea cycle activity in the HLC was not at levels comparable to those we observe in proficient adult hepatocytes. Limited studies exist on UCD and iPSC. In one report, iPSC from an OTCD male were generated but no genetic correction, hepatic differentiation or phenotypic characterization were conducted.[260] In a more circumstantial study, a defect in the *ARG1* gene was repaired in iPSC derived from affected patients and

restored urea cycle.[90] Admittedly, a stronger phenotypic correction upon gene repair could be better shown in cases where the target gene is highly expressed even in fetal hepatocytes; therefore, an adequate expression would be present in most lab-made HLC. A characteristic example could be *A1AT*, a gene that increases earlier in development, and HLC generated in **PAPERS II** and **III** displayed expression in the range of adult hepatocytes. Furthermore, proving phenotypic restoration after genetic correction of a UCD might be additionally difficult since it is a prerequisite that all genes of the cycle are expressed at satisfactory levels, sufficient for ureagenesis measurement.

As mentioned in the introduction section, natural hepatogenesis and liver maturation occur during nine months of prenatal development, and as long as two years after birth receiving countless signals and being exposed to various dynamic changes in the body. Poor maturation of lab-made HLC might be expected if we consider that current differentiation protocols are in most of the cases oversimplified procedures frequently lasting about one month and involving a limited number of factors, cytokines and other molecules. Within the framework of this thesis, we tested if maturation of HLC could occur *in vivo* by transplanting them into FRGN mice; thus, providing them stimuli from a more natural environment. Unfortunately, in the face of many attempts, the cells did not engraft and/or survive. We were able to measure circulating human albumin as low as 10-20 μg/ml which would theoretically correspond to less than 0.1% liver repopulation. Furthermore, we failed to identify the cells in the mouse livers after immunohistochemical analysis, perhaps due to their extremely low number (data not shown). Other studies have shown higher levels of liver reconstitution with human iPSC-HLC, most of them utilizing other animal models than FRGN, such as liver-irradiated Gunn rats reporting liver repopulation up to 7.5%[261] or uPA/SCID mice with up to 20% humanization[262]. Up to date, the efficiency and robustness of iPSC-HLC in repopulating animal livers is far behind that of primary hepatocytes.[141]

Another hypothesis that has been suggested during my doctoral research as a potential solution to the immaturity of HLC was the targeted enhancement or diminution of particular genes crucial for liver development. Our efforts focused on HNF4a, a nuclear receptor proven to be an important regulator of liver development. *HNF4a* encodes several isoforms, originating from distinct promoters and alternative splicing. Some isoforms are mainly present in fetal hepatocytes, while others in mature cells. Therefore, we reasoned that deletion of the fetal promoter through CRISPR or enhancement of expression of mature isoforms through CRISPR activation systems[263] might improve the differentiation. While I consider that such projects constitute great ideas, they were initiated but not continued or terminated because of lack of time. Among other suggestions proposed in the literature to overcome the problem of hepatic immaturity are co-culture of iPSC-HLC with non-parenchymal cells, decellularized livers and CRISPR-based large screenings.[49] I strongly believe that the application of CRISPR-based libraries might disclose genes crucial for the transition from one stage to another during cell differentiation, as well as important genes for cell engraftment and survival in xenogenic settings.

It is generally accepted though that even in the case of incomplete maturation, iPSC-HLC platforms have been recognized to outperform many *in vitro* models, and the currently attained extent of maturation might be sufficient to reproduce some elements of the disease.[78] Furthermore, with the advent of genome engineering, iPSC are easily amenable to genetic manipulations either as a proof of concept, or as an attempt to generate potential sources for cell therapies. It can be stressed that programmable nucleases could be used to correct

pathogenic variants, as well as introduce variants of interest broadening even more the applicability of iPSC. Disease-specific mutations have been introduced in stem cells aiming to mimic phenotypes associated mainly with brain, lung, kidney and intestinal pathologies.[264] Either engineered to bear the mutation or not, iPSC can be of great importance especially given the scarcity of available primary material from patients. In **PAPER V** for instance, because of the limited supply of primary hepatocytes, we generated iPSC from the OTCD patient which were used for the optimization experiments for genome engineering before we proceeded with patient cells.

To our knowledge, **PAPER III** is the first report of iPSC generation from somatic cells derived from OTCD followed by their correction and differentiation into HLC. The reprogramming was mediated through a Sendai virus, an advantageous reprogramming method due to its non-integrating nature. Furthermore, the genetic correction did not rely on any selection method, such as antibiotic or fluorescent marker, maintaining the cells as close as possible to their natural properties. Another advantage of the strategy implemented is the fact that the endogenous *OTC* gene was corrected, instead of introducing a transgene; therefore, the gene continued to be controlled by its endogenous promoter and enhancer. Although, the list of reported pathogenic variants in the *OTC* gene, as well in other urea cycle genes, is quite extended, this universal approach could be easily tailored to identify pathogenic variants in the *OTC* gene and design gRNAs and donor templates for HDR-based genetic manipulations.

A significant part of this thesis focused on the investigation of disease models of UCD. We explored an *in vivo* UCD model through liver-humanization of mice with disease-affected patient hepatocytes, CPS1D in **PAPER IV** and OTCD in **PAPER V**. Briefly, in **PAPER IV**, the presented phenotype by the mice transplanted with CPS1D cells was consistent with clinical manifestations observed in CPS1D patients, including elevated glutamine and glutamate, delayed clearance of ammonia and ureagenesis following a $^{15}NH_4Cl$ infusion. These observations are remarkable, particularly if we consider that the animals were on normal protein diet (19%), without the administration of ammonia scavengers. Therefore, this model could undoubtedly serve the investigators to address molecular, biological and pathological consequences of CPS1D. Importantly, evidence also indicated that other functions of the liver were maintained at normal levels. For instance, OTC enzyme activity and circulating human albumin, as well as similar gene expression levels between the groups transplanted with either CPS1D hepatocyte or proficient cells. Similarly, we showed in **PAPER V** that when mice were liver-repopulated with OTCD hepatocytes, the phenotype of the disease was generated with elevated blood ammonia and urinary orotic acid, as well as decreased OTC enzyme activity in the livers. In summary, we created small animal models of two UCD that faithfully represent the diseases and avoid the challenges that knockout mouse models of UCD possess, including a mild phenotype failing to cover the whole spectrum of symptoms of the disease or high fatality due to their severe deficiency phenotype complicating animal maintenance and making them less amenable to further studies. In addition, this kind of model allows the study of specific mutations generating phenotypes with distinct severities.

The robustness and utility of liver-humanized mice is enhanced by several studies including an early report that demonstrated the ability to repopulate FRG mice with hepatocyte from patients with various metabolic diseases (Maple syrup urine disease, Crigler Najjer, OTCD, familial cholestasis, biliary atresia), but no phenotype characterization was performed.[265]

Recently, a study described the establishment of OTCD mouse model with the transplantation of hemi- and hetero-patient primary hepatocytes.[266] The disease phenotype was recapitulated in the xenografted mice with distinct disease severity between groups that were injected with either hemi- or hetero- OTCD cells indicating the sensitivity of such models. In that study, uPA/SCID mice were used as a mouse of liver humanization, which shares many similarities with the FRGN, but has major difference in the mechanism of hepatotoxicity.

Interspecies differences related to metabolism, cell replication, DNA repair pathways and other aspects might complicate the translation of results from experimental animals to humans. Undoubtedly, studies conducted on liver-humanized mice can provide preclinical evidence that could more easily be extrapolated to human diseases. Cellular and gene therapy, as well as the rapidly emerging gene editing tools could be tested on such platforms. In **PAPER V**, we corrected patient's OTCD cells *ex vivo* and repopulated the livers of the FRGN mice. Notably, we demonstrated that basal ammonia and OTC enzyme activity were completely normalized, exogenous ammonia could be cleared faster by the edited mice when compared to the unedited counterparts, and urinary orotic acid was significantly improved. Surprisingly, while a complete restoration of the disease symptoms might be anticipated in the edited group with editing efficiencies in the range of 80%, not all markers were completely normalized. We observed reduced urea cycle function when the mice were subjected to an ammonia challenge and not fully normalized urinary orotic acid. A potential explanation could be the fact that hepatocytes that repopulated the periportal zone might not have acquired all the zonation-specific functions, including urea cycle. Since full urea cycle activity is limited to hepatocytes in the portal region, edited cells that reside in zones 2 or 3 of the liver would not be expected to contribute to urea cycle, despite having a fully corrected *OTC* gene. Therefore, a problem with any other urea cycle gene could influence the overall cycle activity. Studies investigating the zonation in these models of humanization would be of great interest. Spatial transcriptomics or traditional immunohistochemistry staining against several markers specific for each zone might shed light on this. Another explanation for the discrepancy between editing efficiency on molecular level and the yielded phenotype could be the dominant negative effects for OTC protein observed in hepatocytes that has been described previously.[267] While heterotrimers of mutant and wild-type protein would naturally not be expected, in the case of gene editing this might occur when not all alleles in a cell are corrected. This would lead to the impairment of corrected (wild-type) protein in the presence of heterotrimers underestimating the OTC enzyme activity correction. These hypotheses are worth investigating as they might affect the selection not only of patients but also of diseases subjected to similar therapeutics on clinical level.

Several observations were made during the study in **PAPER V** that have relevance to future applications of CRISPR and other programmable nucleases. First, we demonstrated that human primary hepatocytes can be electroporated and *ex vivo* edited at very high efficiencies. Furthermore, genetically manipulated cells were able to proliferate and contribute proportionally to the unedited counterparts, showing no major selection for, or against the corrected cells. Additional evidence of no adverse effects was provided from the liver pathology investigation which indicated no necrosis, fibrosis or cirrhosis, as well as from the gene expression levels which showed no major differences between mice that received unedited or edited hepatocytes. Finally, exhaustive investigation of potential off-target mutagenesis was performed through CIRCLE-seq platform. The technique identifies unspecific editing events in a sensitive (<0.1%) and unbiased fashion since it relies on patient

specific genome, rather than *in silico* predictions based on reference genomes.[258] After deep sequencing of more than 70 sites that could have been potentially unintentionally edited, we found that none of them was altered; thus, we concluded that *ex vivo* application of CRISPR on human primary hepatocytes was not only effective, but also safe.

In summary, a significant body of scientific research is presented in this thesis demonstrating the utility and versatility of stem cells and liver-humanized mouse models for the creation of platforms for the study of liver genetic diseases. Furthermore, the advent of CRISPR technologies improves these tools, as well as raises potentials for correction of the patient's defective genome. Considerable efforts are required in several fields in order to further accomplish the aims of this thesis, including hepatic differentiation, editing efficiency, especially focusing on methods that would not depend on cell proliferation, as well as more efficient delivery methods. The *ex vivo* genome editing of primary hepatocytes applied in this thesis yielded significant observations for relevant future clinical studies. However, I believe that the most probable scenario for correcting liver (and not only) genetic defects to be translated to humans is *in vivo* genome editing. The father of modern transplantation, Thomas Starzl, wrote "*what was inconceivable yesterday, and barely achievable today, often becomes routine tomorrow.*" Correcting genetic defects directly in the human body might be a prime example. It was inconceivable yesterday, it has become almost achievable today, and it will certainly become a routine tomorrow.

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"Research means that you don't know,
but are willing to find out"
Charles F. Kettering

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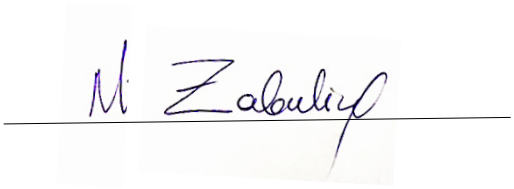

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