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FIGHTING CARDIOMETABOLIC DISEASE: VALIDATION OF NEW EXPERIMENTAL MODELS AND THERAPEUTIC TARGETS

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Fighting cardiometabolic disease: validation of new experimental models and therapeutic targets
THESIS FOR DOCTORAL DEGREE (Ph.D.)

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This is water

David Foster Wallace

ABSTRACT

The prevalence of cardiometabolic diseases (CMD) such as atherosclerotic cardiovascular diseases, type 2 diabetes mellitus and nonalcoholic fatty liver disease (NAFLD), has grown dramatically during the last decades. Hence, massive research efforts are allocated to identify the pathophysiological mechanisms and new therapeutic targets for these morbidities. However, the data gained from preclinical studies using *in vitro* cellular or *in vivo* animal models are not always clinically translatable. The overall aim of this thesis was to develop and characterize new experimental models relevant to the human condition with respect to liver and lipoprotein metabolism, and to use these models to validate new therapeutic targets to treat CMD.

Various strains of mice, genetically altered or unaltered, are extensively used to study human CMD. However, major species differences limit the human translatability of animal models. In **Papers I** and **II** we thoroughly characterized the lipoprotein and liver metabolism of liver-humanized mice (LHM), a promising preclinical model to study human hepatic metabolism. To generate LHM, immunocompromised *Fah*/*Rag2*/*Il2rg*-triple knockout mice on the nonobese diabetic background are repopulated with human hepatocytes. Cholesterol lipoprotein profiles of LHM showed a human-like pattern, shifting the cholesterol transport into low-density lipoprotein (LDL) rather than in high-density lipoprotein particles. The humanization of lipoprotein profiles does not require cholesteryl ester transfer protein, and was instead determined by higher levels of apolipoprotein B100 in the circulation, as a result of lower hepatic mRNA editing and LDL receptor expression, and higher levels of circulating proprotein convertase subtilisin/kexin type 9. As a consequence, LHM lipoproteins bind to human aortic proteoglycans in a pattern similar to human lipoproteins, which entails the potential use of LHM as a model for studies of atherosclerosis. A human-like bile acid metabolism was also observed in LHM, with higher levels of glycine-conjugated bile acids and taurodeoxycholic acid, and lower levels of mouse-specific tauromuricholic acids. However, an altered enterohepatic signaling in LHM results in abnormal bile acid synthesis. We also investigated the response to pharmacological and dietary stimuli in LHM. When treated with the liver X receptor (LXR) agonist GW3965, LHM mimicked the negative lipid outcomes seen in the first human trial of LXR stimulation, and thus allowed the characterization of the hepatic effects at a molecular level. To induce CMD in mouse models, challenge with high-fat/high-sucrose diet (HFHSD) is often used. However, LHM appeared to be resistant to HFHSD. We also present the preliminary results on the development of sever hepatic steatosis and atherosclerosis after feeding LHM with a high-fat/high-fructose/high-cholesterol diet. Taken together, these results indicate LHM as an interesting translatable model of human hepatic and lipoprotein metabolism. Because several metabolic parameters displayed donor dependency, LHM may also be used for studies of personalized medicine.

Human hepatocyte-like cell lines (such as HepG2, Huh7 and Huh7.5 cells) are also widely used in preclinical research to study CMD. However, these cell lines exhibit major

differences compared with human hepatocytes *in vivo*. For instance, hepatocytes in *vivo* only express sterol-O acyltransferase (*SOAT*) 2, whereas both *SOAT1* and *SOAT2* are found in HepG2, Huh7 and Huh7.5 cells. *SOAT1* and *SOAT2* catalyze the formation of cholesteryl esters, but only *SOAT2* determines the amount of CE secreted in apolipoprotein B-containing lipoproteins. Therefore, in **Paper III** we used the clustered regularly interspaced short palindromic repeats (CRISPR) technology to knock out *SOAT1* in HepG2 and Huh7.5 cells. Moreover, culturing HepG2 cells with medium supplemented with human instead of fetal bovine serum dramatically improves the lipid and lipoprotein metabolism. Hence, unedited and *SOAT2*-only cells were cultured with either fetal bovine or human serum to assess whether the combination of *SOAT1*-KO with culturing with human serum could additionally improve the phenotype of HepG2 and Huh7.5 cells. *SOAT2*-only-HepG2 cells exhibited higher levels of cholesterol, triglycerides and apolipoprotein B in the medium compared with unedited HepG2 cells. Further increase was seen when culturing *SOAT2*-only-HepG2 cells with human serum. Opposite effects were instead found in *SOAT2*-only-Huh7.5 cells. This study shows that *SOAT1* expression in hepatocyte-like cells contributes to the distorted phenotype observed in HepG2 and Huh7.5 cells. *SOAT2*-only-HepG2 cells cultured with human serum represent an improved model for studies of human hepatic lipid metabolism.

Inhibition of the lipid droplet-associated gene cell death-inducing DFFA-like effector c (*CIDEC*) has been proposed as a therapeutic strategy for hepatic steatosis and NAFLD. Hence, in **Paper IV** we knocked out *CIDEC* in HepG2 cells using the CRISPR technology in order to study its potential role as therapeutic target for hepatic steatosis/NAFLD. Knockout of *CIDEC* in HepG2 cells was accompanied by changes in the expression of several mediators of lipid metabolism. Nonetheless, the intracellular levels of cholesterol and triglycerides were not affected. Future studies will elucidate the role of *CIDEC* in hepatic lipid and carbohydrate metabolism and its potential as a therapeutic target for hepatic steatosis.

Collectively, these results highlight LHM and *SOAT2*-only-HepG2 cells cultured with human serum as new preclinical models that greatly improve the translatability into humans compared with the commonly used *in vivo* and *in vitro* models.

LIST OF SCIENTIFIC PAPERS

1. **Minniti ME**, Pedrelli M, Vedin LL, Delbès AS, Denis RGP, Öörni K, Sala C, Pirazzini C, Thiagarajan D, Nurmi HJ, Grompe M, Mills K, Garagnani P, Ellis ECS, Strom SC, Luquet SH, Wilson EM, Bial J, Steffensen KR, Parini P. Insights from liver-humanized mice on cholesterol lipoprotein metabolism and LXR-agonist pharmacodynamics in humans. *Hepatology*, 2019.

2. **Minniti ME**, Pedrelli M, Vedin LL, Delbès AS, Denis RGP, Sala C, Filippi C, Dhawan A, Garagnani P, Wilson EM, Bial J, Luquet SH, Parini P. Are liver-humanized mice relevant to study the high-fat/high-sucrose diet challenge? *Manuscript*

3. Pramfalk C, Jakobsson T, Verzijl CRC, **Minniti ME**, Obensa C, Ripamonti F, Olin M, Pedrelli M, Eriksson M, Parini P. Generation of new hepatocyte-like *in vitro* models better resembling human lipid metabolism. *Biochim Biophys Acta Mol Cell Biol Lipids*, 2020; 1865(6):158659.

4. **Minniti ME**, Jakobsson T, Pramfalk C, Verzijl CRC, Cricrì D, Eriksson M, Parini P. A new hepatocyte-like cell model to study the inhibition of cell death-inducing DFFA-like effector c in liver lipid and carbohydrate metabolism. *Manuscript*

CONTENTS

LIST OF ABBREVIATIONS

ABC	ATP-binding cassette
ANOVA	Analysis of variance
APO	Apolipoprotein
APOBEC1	APOB mRNA editing enzyme catalytic subunit 1
ASCVD	Atherosclerotic cardiovascular diseases
ATP	Adenosine triphosphate
BA	Bile acid
C4	7alpha-hydroxy-4-cholesten-3-one
CA	Cholic acid
cAMP	3',5'-cyclic adenosine monophosphate
Cas9	CRISPR-associated protein 9
CD	Cluster of differentiation
cDNA	Complementary DNA
CE	Cholesteryl ester
CEC	Cholesterol efflux capacity
CETP	Cholesteryl ester transfer protein
CIDE	Cell death-inducing DFFA-like
CMD	Cardiometabolic diseases
Cpt-cAMP	8-(4-chlorophenylthio)-cAMP
CRISPR	Clustered regularly interspaced short palindromic repeats
CYP	Cytochrome P450
DCA	Deoxycholic acid
DFFA	DNA-fragmentation factor subunit alpha
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
Fah	Fumarylacetoacetate hydrolase
FBS	Fetal bovine serum
FC	Free (or unesterified) cholesterol
FGF	Fibroblast growth factor
FRG-KO	*Fah*/*Rag2*/*Il2rg* triple KO

FRGN	FRG-KO on NOD background
FSP27	Fat-specific protein 27
FXR	Farnesoid X receptor
GC-MS	Gas chromatography-MS
GO	Gene Ontology
gRNA	Guide RNA
H&E	Hematoxylin and eosin
haPG	Human aortic PG
HDL	High-density lipoprotein
HFHSD	High-fat/high-sucrose diet
HMGC	3-hydroxy-3-methylglutaryl-coenzyme A
HMGCR	HMGC reductase
HS	Human serum
HSD	Honestly significant difference
HUMAN	Health and the Understanding of Metabolism, Aging and Nutrition
IDL	Intermediate-density lipoprotein
Il2rg	Interleukin 2 receptor, gamma chain
KO	Knockout
LC-MS/MS	Liquid chromatography-tandem MS
LDL	Low-density lipoprotein
LDLR	LDL receptor
LHM	Liver-humanized mice
LIPC/HL	Lipase C, hepatic type/Hepatic lipase
LMM	Liver-murinized mice
Lp(a)/*LPA*	Lipoprotein(a)
LPL	Lipoprotein lipase
LXR	Liver X receptor
MCA	Muricholic acids
mRNA	Messenger RNA
MS	Mass spectrometry

NAFLD	Nonalcoholic fatty liver disease
NASH	Nonalcoholic steatohepatitis
NASH-diet	High-fat/high-fructose/high-cholesterol diet
NEFA	Non-esterified fatty acids
NOD	Nonobese diabetic
NPC1L1	Niemann-Pick C1-like intracellular cholesterol transporter 1
OCT	Optimal cutting temperature
ORO	Oil Red O
PCR	Polymerase chain reaction
PCSK9	Proprotein convertase subtilisin/kexin type 9
PG	Proteoglycans
PHH	Primary human hepatocytes
PL	Phospholipids
PLTP	Phospholipid transfer protein
PPAR	Peroxisome proliferative-activated receptor
qPCR	Quantitative real-time PCR
Rag2	Recombination activating gene 2
RCT	Reverse cholesterol transport
RNA	Ribonucleic acid
rRNA	Ribosomal RNA
SEC	Size-exclusion chromatography
SEM	Standard error of the mean
SOAT	Sterol O-acyltransferase
SRB1	Scavenger receptor class B member 1
SREBF	Sterol regulatory element-binding transcription factor
SREBP	Sterol regulatory element-binding protein
T2D	Type 2 diabetes mellitus
TC	Total cholesterol
TG	Triglyceride
VLDL	Very low-density lipoprotein

1 BACKGROUND

1.1 CARDIOMETABOLIC DISEASES

Cardiometabolic diseases (CMD) are defined as a cluster of metabolic disorders leading to atherosclerotic cardiovascular diseases (ASCVD) and type 2 diabetes mellitus (T2D).[1] Several metabolic diseases are strongly associated with atherosclerosis and cardiovascular risk, including visceral obesity, hypertension, dyslipidemia, insulin resistance and nonalcoholic fatty liver disease (NAFLD).[2-4] Moreover, progression of atherosclerosis leads to the most common form of ASCVD, and is promoted by an impaired lipid metabolism.[2, 4, 5] Over the last fifty years, the prevalence of CMD has grown dramatically due to the increase of life expectancy and cultural transitions in lifestyle and nutrition – also in low- and middle-income countries – causing a massive economic burden for the healthcare systems worldwide.[3, 4, 6] According to the mortality estimates from World Health Organization (WHO) outlined in Figure 1.1, in 2000 around 14 million deaths worldwide were caused by CMD (*i.e.*, ischemic heart disease, stroke, T2D and hypertensive heart disease), corresponding to 26% of total deaths.[7] By the year 2016, deaths caused by CMD increased to around 18 million (31% of total deaths) (Figure 1.1).[7] As a confirmation of this interrelationship, CMD and ASCVD share similar risk factors, classified in (a) behavioral (smoking, physical inactivity, unbalanced diet, and excess of alcohol), (b) metabolic (dyslipidemia, hypertension, insulin resistance, and obesity), and (c) others (age, sex, genetic disposition, stress, poverty, and low education).[2]

According to WHO, there is strong evidence that behavioral and metabolic risk factors play a key role in the etiology of ASCVD (and thus CMD).[2, 4] Therefore, the first-line intervention to prevent ASCVD and mitigate the cardiometabolic risk consists of a healthy lifestyle that includes a low-fat diet and regular physical activity.[2, 4]

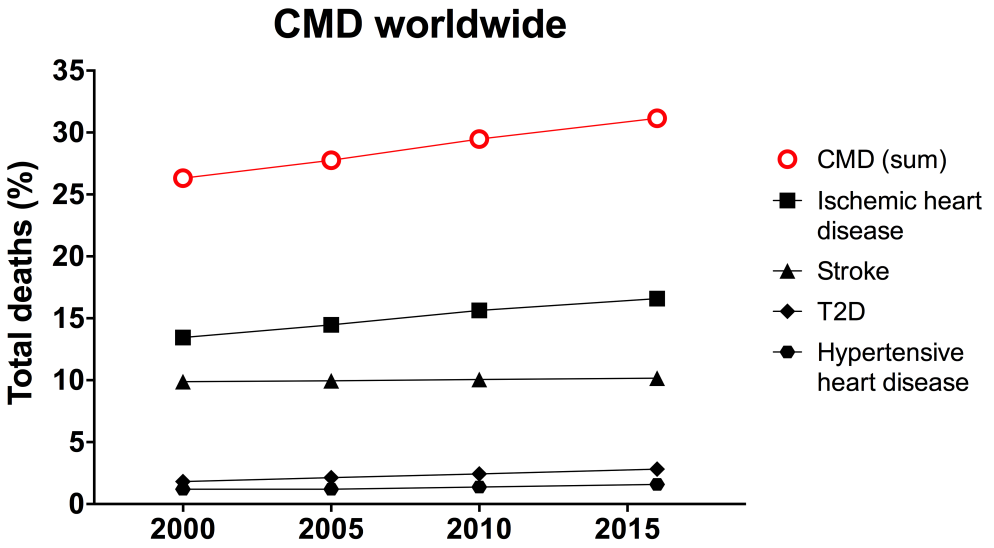

Figure 1.1 Estimates of worldwide deaths by CMD in 2000-2016. *Data from Global Health Estimates 2016 from the WHO.[7] CMD (in red) represents the sum of the total death estimates from ischemic heart disease, stroke, T2D and hypertensive heart disease.*

1.1.1 Lipoproteins and atherosclerotic cardiovascular diseases

Lipids, such as triglycerides (TGs) and cholesterol, are fundamental biological molecules. However, impaired lipid homeostasis in the organism represents a critical risk factor for CMD.[2, 4] Because lipids are insoluble in water, they are packaged with protein components named apolipoproteins (APOs) in lipoprotein complexes for transportation in the bloodstream. There are multiple classes of APOs, which determine the structural and functional properties of the particles, affect plasma uptake and clearance, and serve as enzyme activators or inhibitors.[4] Lipoproteins are commonly classified by their density, and can further be divided in two main groups containing either APOB or APOA1. APOB is present in all atherogenic particles, *i.e.*, chylomicrons, chylomicron remnants, very low-density lipoproteins (VLDLs), intermediate-density lipoproteins (IDLs), low-density lipoproteins (LDLs), and lipoprotein(a) (Lp(a)).[4] APOA1-containing particles include high-density lipoproteins (HDLs), which are considered atheroprotective due to their functionalities (*e.g.*, anti-inflammatory and antioxidant activity and cholesterol efflux capacity (CEC)).[4, 8]

The atherogenicity of APOB-containing lipoproteins is explained by the interaction of APOB with sub-endothelial (or intima) proteoglycans (PG).[5] APOB binding retains the lipoprotein particle in the arterial wall,[5] triggering the first step in early atherogenesis according to the "response-to-retention" hypothesis.[9, 10] Because large chylomicron and VLDL particles cannot penetrate the endothelium – in contrast to their remnants and LDLs – the size of the particle is determining its retention within the intima.[4] Therefore, the main traditional risk factor for ASCVD in the general population is represented by high plasma levels of LDL-cholesterol.[4] In 2017, the overall evidence from genetic, epidemiological, Mendelian randomization, and randomized trials of LDL-cholesterol-lowering therapies, led a consensus panel from the European Atherosclerosis Society to state that LDL-cholesterol is causative of ASCVD.[4, 11, 12]

Nonetheless, in individuals with metabolic disorders such as T2D, high plasma levels of TGs (or TG-rich lipoprotein remnants) and low HDL-cholesterol also increase the cardiometabolic risk.[4] These two features are often associated with one another, and low HDL-cholesterol levels have been shown to be a strong risk factor for ASCVD.[4] However, genetic studies suggest that HDL-cholesterol has no role for ASCVD, whereas elevated remnant cholesterol (*i.e.*, the cholesterol contained in TG-rich lipoproteins) is a causal risk factor for ASCVD.[4, 13] Furthermore, both LDL-cholesterol and remnant cholesterol are causally associated with ASCVD, but only remnant cholesterol is also causally associated with low-grade inflammation.[13] This suggests TGs to prompt the inflammatory component in the atherosclerosis process.

1.1.2 Liver and cardiometabolic diseases

The liver has a key role in the regulation of cholesterol and lipoprotein homeostasis. Cholesterol is a fundamental biological molecule, being a crucial constituent of biological membranes where it promotes proper membrane fluidity and permeability, and it produces "rafts" involved in endocytosis and cell signaling.[14] Moreover, cholesterol is precursor of bile

acids (BAs), oxysterols (*i.e.*, oxygenated derivatives of cholesterol), steroid hormones and vitamin D, which can act as regulators of the transcriptional response and affect several vital functions. Cholesterol has also a role during the embryonic development, as is used as a substrate to modify covalently several signaling mediators (*e.g.*, hedgehog proteins). Alterations of the cholesterol biosynthesis, both during the development and in the adult state, may lead to severe malformations and diseases.[15]
As comprehensively outlined,[16] cholesterol (as many lipids) can be both produced *de novo* (endogenous pathway) or absorbed in the intestine, the latter coming from the diet or the enterohepatic circulation (exogenous pathway). Excretion pathways maintain the balance between the synthesis and the absorption of cholesterol.

1.1.2.1 Endogenous pathway

The biochemical pathway for the synthesis of cholesterol was elucidated by Bloch in the 1960s.[17] Cholesterol biosynthesis begins with the condensation of one molecule of acetyl-coenzyme A and one molecule of acetoacetyl-coenzyme A to produce 3-hydroxy-3-methylglutaryl-coenzyme A (HMGC) by HMGC synthase. HMGC is reduced to mevalonate by the rate-limiting enzyme HMGC reductase (HMGCR), anchored to endoplasmic reticulum membrane.[18] Mevalonate is then transformed into isopentenyl pyrophosphate, which is the substrate for subsequent polymerizations and modifications leading to the synthesis of cholesterol.
In human hepatocytes, free (or unesterified) cholesterol (FC) is transported to the endoplasmic reticulum, and is esterified with fatty acyl-coenzyme A by sterol O-acyltransferase (SOAT) 2. Cholesteryl esters (CEs) can then be stored in lipid droplets inside the cell or packed together with TGs, phospholipids (PL), FC, and APOB100 and secreted in the bloodstream as nascent VLDLs. In the circulation, VLDL particles mature by acquiring other APOs such as APOE and APOCs: APOE serves as ligand mainly for the LDL receptor (LDLR)-related protein 1 (LRP1), allowing the uptake of large and buoyant particles from the bloodstream, whereas APOCs affect the lipolysis of the TGs contained in the particle.[19]
VLDLs are rapidly taken up via the VLDL receptor (VLDLR), which is widely expressed in adipose tissues, heart, muscle, and endothelial cells (but with very low expression in liver).[20] In these tissues, the VLDL uptake leads to the upregulation of lipoprotein lipase (LPL), which hydrolyzes VLDL TGs into glycerol and non-esterified fatty acids (NEFA). LPL is mainly produced by the adipose, heart and muscle tissue, and it is active at the luminal surface of the capillary endothelium of the tissue of origin.[21] NEFA enter the tissues to be used for energy (muscle) or storage (adipose), or bind to serum albumin to be carried throughout the systemic circulation. The decrease in TG content modifies the size and the density of VLDLs, which turn into IDLs. The TGs contained in IDLs are mostly hydrolyzed by lipase C, hepatic type (LIPC, also known as hepatic lipase, HL), which is produced by the liver and it is active in the hepatic endothelia.[22] LIPC/HL transforms IDLs in TG-poor and cholesterol-rich LDLs, responsible for the movement of cholesterol mainly to peripheral tissues. Also, plasma lipid transfer proteins, which include the cholesteryl ester transfer protein (CETP) and the phospholipid transfer protein (PLTP), affect lipoprotein concentration

and composition by mediating the transfer of lipids between the different lipoprotein particles. CETP exchanges CEs in HDLs for TGs contained in VLDLs or LDLs, whereas PLTP transfers surface PL from TG-rich particles to HDLs.[23] The liver can take up IDLs and LDLs mainly via the LDLR.

FC and PL can also be secreted to APOA1 by the transporter adenosine triphosphate (ATP)-binding cassette (ABC) A1, which is expressed on the basolateral membrane of hepatocytes and enterocytes, as well as in other tissues. This step is fundamental for the formation of HDLs,[24] responsible for moving cholesterol from the periphery back to the liver via the reverse cholesterol transport (RCT) pathway. In the bloodstream, the lecithin-cholesterol acyltransferase (LCAT) esterifies the FC in nascent pre-beta HDLs, contributing to the maturation of HDL particles.[25] PLTP, CETP and LIPC/HL take also part to HDL remodeling.[22, 23, 26] Furthermore, HDL particles can exchange APOs and incorporate other lipids, thus increasing in size. The hepatocyte takes up the cholesterol in mature HDLs mainly via the bidirectional transporter scavenger receptor class B member 1 (SRB1).[26]

1.1.2.2 Exogenous pathway

Cholesterol absorbed in the intestine derives from several sources, including diet, bile, intestinal secretion and epithelial cell sheading. Absorption of dietary CEs requires hydrolysis and emulsification with BAs to form micelles that are taken up by the enterocytes. Cholesterol uptake is facilitated by Niemann-Pick C1-like intracellular cholesterol transporter 1 (NPC1L1), a transporter located on the apical brush border membrane of enterocytes in the proximal small intestine.[27] Intestinal cholesterol uptake is antagonized by the obligated heterodimer of ABCG5:ABCG8, expressed on the apical membrane of enterocytes where it facilitates the efflux of FC back to the intestinal lumen.[28]

Similarly to hepatocytes, enterocytes can synthesize cholesterol via HMGCR. Moreover, both endogenous and exogenous FC is esterified by SOAT2. FC and CEs are incorporated together with TGs, PL and APOB48 into chylomicrons. These lipoproteins are secreted into the lymphatic circulation to reach the bloodstream, and transport the dietary lipids to peripheral tissues. Here, chylomicrons follow the same fate as VLDLs: they maturate and exchange lipids and APOs with other lipoproteins, bind to VLDLR and their TG content is hydrolyzed by LPL.[29] These modifications lead to the formation of chylomicron remnants, particles rich in cholesterol and APOE, that can be taken up by the liver via LDLR or LRP1.

1.1.2.3 Cholesterol excretion

In humans, cholesterol cannot be catabolized to produce energy. Therefore, fecal excretion is the predominant way for its disposal. The liver excretes the excess of FC into the bile as such via ABCG5:ABCG8, which is expressed on the hepatocyte canalicular membrane,[30] or after its conversion into more soluble BAs. Cytochrome P450 (CYP) 7A1 and CYP27A1 are the main rate-limiting enzymes that convert FC to BAs.[31] As previously reviewed,[32, 33] CYP7A1 and CYP8B1 are involved in the classical or neutral pathway, which is quantitatively more important and favors the synthesis of cholic acid (CA). CYP27A1 and CYP7B1 mediate the alternative or acidic pathway, which favors the synthesis of chenodeoxycholic acid in

humans, and is present also in extra-hepatic tissues, such as macrophages. In addition, the classical and alternative pathways can converge in certain reactions. The BAs synthesized in the liver (primary BAs) are conjugated with either glycine or taurine before secretion into the gallbladder bile via ABCB11. Humans express NPC1L1 in the bile canaliculi, where it seems to mediate the reuptake of biliary FC.[34, 35] Primary BAs are partly dehydroxylated into secondary BAs by the action of the gut microbiota. Through enterohepatic circulation, BAs are resorbed and recycled in the liver. High levels of BAs in the intestine can activate farnesoid X receptor (FXR), which leads to secretion of fibroblast growth factor (FGF) 19 (in humans, FGF15 in mice). In hepatocytes, both FXR activation and FGF19 binding inhibit the BA synthesis. BA homeostasis is therefore maintained by enterohepatic circulation.
Cholesterol can also be excreted into the feces by a non-biliary pathway known as trans-intestinal cholesterol excretion (TICE). However, substantial cholesterol secretion from the small intestine has been observed only in non-primate animal models. The presence and the extent of TICE in humans still require further investigations.[36]

1.1.2.4 Regulation of cholesterol homeostasis

HMGCR is a key enzyme in the regulation of cholesterol homeostasis. It is highly regulated through several mechanisms both at transcriptional and post-transcriptional levels.[37-40] The intracellular amount of FC and oxysterols regulate the HMGCR turnover via the sterol regulatory element-binding protein (SREBP) 2, a transcription factor that affects the expression of many genes involved in lipid metabolism. The role of SREBP2 was established by Goldstein and Brown in the 1990s.[41] After its synthesis, SREBP2 binds to SREBP cleavage-activating protein (SCAP) in the endoplasmic reticulum. Decrease in the sterol levels results in the transport of SCAP-SREBP2 complex to the Golgi, where SREBP2 is processed by two serine proteases to release a transcriptionally active N-terminal fragment. This fragment migrates to the nucleus, where it binds the sterol regulatory element upstream of target genes (*e.g.*, *HMGCR*, *LDLR*) and activates the transcription. The opposite, increase in the sterol levels leads to the retention of the SCAP-SREBP2 complex.
Oxysterols seem also to directly regulate cholesterol homeostasis. Although they are present at very low physiological levels compared with cholesterol, in biological systems where the ratio of the oxysterols to cholesterol is higher than 1:1000 (*e.g.*, brain and cholesterol-loaded macrophages) they may activate liver X receptors (LXRs).[42] LXR alpha and beta differ for the tissue expression patterns, and are able to induce the elimination of cholesterol by increasing the expression of target genes involved in cholesterol efflux (*e.g.*, *ABCA1*, *ABCG1*).[43] In addition, LXR activation in mouse liver (but not in humans) increases BA synthesis via *Cyp7a1*.[44]

Under physiological conditions, cholesterol represents the largest quota of lipids being endogenously synthesized.[45] Although cholesterol can be synthesized by most cells in the body, the liver (and intestine, but to a lesser extent) produces most of the cholesterol. Furthermore, only the liver and intestine secrete APOB-containing lipoproteins, thus

distributing TGs and cholesterol to the peripheral tissues. For these reasons, the liver and intestine are the main targets of dyslipidemia pharmacotherapy.[4, 46] When lifestyle intervention is not sufficient to reduce ASCVD/CMD risk, the first-choice treatment is the use of HMGCR inhibitors – commonly known as statins – which block the rate-limiting step in cholesterol biosynthesis.[4, 17] Low intracellular levels of FC activate the SREBP2 pathway, which in turn induces the expression of *LDLR* and the uptake of LDLs from plasma.[4, 41] Nonetheless, lifestyle and statin intervention, separately or combined, are not always able to completely eliminate the cardiometabolic risk.[4, 47]

Perturbations of lipid metabolism can also induce abnormal lipid accumulation (or steatosis) in the liver. Hepatic steatosis is indicated as the initial stage of NAFLD when occurring without secondary causes such as excessive alcohol consumption, medications, or genetic and viral diseases.[48, 49] NAFLD is the most common form of chronic liver disease and is associated with metabolic comorbidities such as obesity, T2D and dyslipidemias.[48, 49] Moreover, it worsens glycemic control in subjects with T2D, contributes to the development and progression of T2D itself and of the most important complications, including ASCVD and chronic kidney disease.[48, 49] Therefore, NAFLD has been widely accepted as the hepatic manifestation of CMD.[48, 49] During hepatic steatosis, significant accumulation of lipids principally as TGs and CEs within cytoplasmic lipid droplets occurs.[48, 49] Chronic combination of hepatic steatosis with low-grade inflammation is defined as nonalcoholic steatohepatitis (NASH), which is characterized by hepatocyte damage (*e.g.*, ballooning) with or without fibrosis.[48, 49] NASH can eventually progress to cirrhosis, liver failure and liver cancer, and is an increasing indication for liver transplantation.[48, 49]

1.1.2.5 Cell death-inducing DFFA-like effector c

Among the recently-found targets involved in CMD, growing evidence has shown that lipid-droplet proteins play a role in the pathophysiology of hepatic steatosis and NAFLD.[50-52] Cell death-inducing DFFA-like (CIDE) effector c (*CIDEC*) and its mouse orthologous fat-specific protein 27 (*Fsp27*) belong to the CIDE family and encode for a lipid-droplet-associated protein highly expressed in normal condition in white and brown adipose tissues. Fsp27/CIDEC is enriched at the sites of contact of two pairing lipid droplets, and promotes the fusion of small droplets into larger ones by facilitation of lipid transfer.[53] Among the other CIDE proteins, CIDEA and CIDEB share similar structure and function, and are highly expressed in white adipose tissue and the liver, respectively.[54] *Fsp27*/*CIDEC* is expressed at low levels in normal liver, but highly expressed in the liver of animal models of obesity and hepatic steatosis, or following feeding the animals a high-fat diet.[50, 55, 56] Hepatocyte-specific *Fsp27* knockdown in diabetic mice ameliorates hepatic steatosis, whereas hepatocyte-specific overexpression induces lipid-droplet synthesis, represses mitochondrial β-oxidation, and decreases TG turnover.[50] Moreover, partial silencing of *Fsp27* with antisense oligonucleotides in wildtype or diabetic mice fed chow or high-fat diets has been shown to improve insulin sensitivity and whole-body glycemic control,[57] and even to reduce atherosclerosis in *Ldlr*$^{-/-}$ mice.[58] However, mice with whole-body or adipocyte-specific disruption of *Fsp27* fed a high-fat diet exhibited increased lipolysis, hepatic steatosis and

insulin resistance, as consequence of the massive influx of NEFA.[59, 60] Hence, only hepatic Fsp27/CIDEC should be targeted to prevent adipose tissue lipodystrophy and ectopic lipid accumulation. Furthermore, peroxisome proliferator-activated receptor (PPAR) gamma (PPARG) was found to control *Fsp27* in diabetic mice, as liver-specific ablation of *Pparg* markedly suppressed *Fsp27* expression.[50] However, in human and mouse liver PPARG is expressed at only 10–30% of the levels found in adipose tissue,[61] and its expression dramatically increases in fatty liver of diabetic mice.[62] In addition, various transcriptional regulators have been reported to mediate *Fsp27/CIDEC* expression in the liver, including PPAR alpha (PPARA, which seems to control *Fsp27*/*CIDEC* expression in normal liver),[63] LXR alpha,[64] SREBP1c,[65] and several 3',5'-cyclic adenosine monophosphate (cAMP)-responsive element-binding proteins (CREBs).[66, 67] These intricate regulation networks underline the difficulties in identifying new pharmacodynamic targets and the need to deepen our knowledge in the field.

1.1.3 Genetic disposition to cardiometabolic diseases

As aforementioned, behavioral and metabolic risk factors are the first intervention target for ASCVD and CMD. However, also non-modifiable risk factors such as sex, age and genetic disposition contribute to the pathophysiology of CMD.[2] It is well known that a number of mutations in genes involved in cholesterol metabolism, such as *LDLR*, *APOB* and proprotein convertase subtilisin/kexin type 9 (*PCSK9*, a posttranslational negative regulator of LDLR in the circulation), cause severe hyperlipidemia.[4, 68] In addition, several genome-wide association (GWAS) studies have identified a plethora of genetic variants considered as risk factors for CMD. Among the genes reported to be associated to CMD are for instance *APOE*,[69-72] transcription factor 7-like 2 (*TCF7L2*),[73] alpha-ketoglutarate-dependent dioxygenase FTO (*FTO*),[74] melanocortin 4 receptor (*MC4R*),[75, 76] and patatin-like phospholipase domain-containing 3 (*PNPLA3*).[77]

1.1.4 Differences in liver metabolism between human and mouse

In the last decades, massive research efforts have been allocated to identifying the pathophysiological mechanisms of metabolic disorders and ASCVD. Nonetheless, the results gained from preclinical studies using animal models are not always translatable to humans, especially due to vast differences in liver metabolism even within mammals.[78] Among the different vertebrate and invertebrate animal models used in research, mice represent the most common model for the phylogenetic closeness to humans, availability, size, easy housing and handling, fast reproduction rate, and the fact that they can relatively easily be genetically modified by various methods. Different strains of mice, genetically altered or unaltered, have been indeed used as translational platforms.[45, 79] In addition, sex differences have a great impact on the susceptibility to CMD, even in mice with the same genotype.[79]
In both humans and mice, the liver is the center of lipid biosynthesis,[78] but the two species differ in lipoprotein and BA metabolism, in the susceptibility to develop ASCVD/CMD, in the response to the most common lipid-lowering drugs (*e.g.*, statins and LXR stimulation), and show opposite sex-related differences with respect to hepatic lipid and BA metabolism

(*e.g.*, BA synthesis, cholesterol esterification, HDL synthesis). Some of the most important differences in cholesterol, lipoprotein and BA metabolism between humans and mice can be summarized as follows:

- plasma cholesterol is mainly transported in LDLs in humans, but in HDLs in rodents;
- humans have lower rates of cholesterol synthesis and dietary intake, compared with mice.[45] Moreover, the hepatic clearance of LDLs is higher in mice;[45]
- editing of the *APOB*/*Apob* mRNA mediated by APOB mRNA editing enzyme catalytic subunit 1 (APOBEC1), which produces APOB48 instead of APOB100, occurs only in the intestine in humans but also in the liver in rodents;[80]
- humans exhibit CETP activity in plasma, conversely to rodents;[81, 82]
- humans – but not rodents – express the Lp(a) gene (*LPA*) in the liver and have circulating Lp(a) in plasma;[83]
- humans have lower plasma lipolytic activity. LIPC/HL is bound to the endothelium membranes in humans, but circulates in the bloodstream in mice.[84] Moreover, LPL is highly expressed in adipose tissue and heart in both humans and mice, but seems also to be produced in the liver by adult mice;[85]
- humans express *NPC1L1* in the liver and intestine, whereas NPC1L1 is found only in the intestine in mice;[34]
- the rate of BA synthesis in the liver is lower in humans compared with mice. In addition, mouse *Cyp7a1* – but not human *CYP7A1* – is an LXR-target gene;[33, 44, 86]
- humans do not synthesize via the alternative pathway the 6-hydroxylated muricholic acids (MCA), which represent instead the largest quota of the mouse BA pool;[87-90]
- in humans, amidation with glycine rather than taurine is used to conjugate BAs, conversely to mice;[33, 86]
- humans cannot rehydroxylate deoxycholic acid (DCA) to CA, in contrast to rodents.[33, 86]

1.1.5 Mouse and diet models to study cardiometabolic diseases

Despite the limitations in using animal models to gain insights on human metabolism due to major species-specific differences, challenging animal models with high-fat or high-fat/high-sucrose diet (HFHSD) is frequently used in studies of the pathogenic process leading to CMD.[91] As a matter of fact, the standard chow diet (5% fat and 0.02% cholesterol in weight) does not induce hyperlipidemia in most rodent models used in studies of liver and lipoprotein metabolism. C57BL/6 mice, one of the most commonly used mouse strains in animal research, carry most of the cholesterol in HDLs and are very efficient in clearing TG-rich lipoproteins also when fed a high-fat/low-cholesterol diet. C57BL/6 mice are also resistant to develop hyperlipidemia when fed HFHSD,[92, 93] but exhibit a mild NASH phenotype after around 6 months on this diet.[94] Because cholesterol enrichment in the APOB-containing lipoproteins is necessary for the atherogenic process, mouse models of atherosclerosis are usually genetically modified. For studies of atherosclerosis, mice knocked out for the *Ldlr* or *Apoe* are commonly challenged with a high-fat/high-cholesterol diet, although *Apoe*$^{-/-}$ mice

do also develop atherosclerosis when fed a chow diet.[79] Yet the particles predominantly accumulated in the *Apoe*$^{-/-}$ mice are chylomicrons or VLDL remnants rich in cholesterol and containing APOB48,[79] thus with little relevance for the human pathophysiology. *Apobec1*$^{-/-}$ and APOB100-only mice produce only APOB100, and attain a human-like profile only on the *Ldlr*$^{-/-}$ background, thus displaying severe hypercholesterolemia.[95, 96] CETP-transgenic mice exhibit an increase in LDL-cholesterol, or VLDL-cholesterol when bred with *Apobec1*$^{-/-}$ or *Ldlr*$^{-/-}$, however without showing a human-like lipoprotein profile.[97, 98] It is also worth mentioning that adult humans have higher levels of LDL-cholesterol even on a low-fat diet. Considering the increase in plasma lipids as a result of dietary challenge, a diet enriched in fat is a prerequisite in studies of lipoprotein metabolism and related diseases in most animal models, and yet these models show little relevance to the human condition.

1.2 EMERGING *IN VIVO* MOUSE MODELS TO STUDY CARDIOMETABOLIC DISEASES

To overcome these limitations, the use of chimeric mice engrafted with human cells represented a prominent approach. The generation of these models requires use of immune-deficient mice and a selective growth advantage for the human cells over the resident ones (for review see [99]).

1.2.1 Liver-humanized mice

In the context of CMD and liver metabolism, the use of chimeric mice harboring human hepatocytes designated an important development in models available for preclinical research.[99] Several liver-humanized mouse (LHM) models have been developed during the latest twenty years, all based on the same principle, *i.e.*, a hepatotoxic treatment directed against the mouse hepatocytes that allows the repopulation of the engrafted human cells.[99] The first model developed in the 2000s was the albumin-uroplasminogen activator (Alb-uPA) transgenic mouse.[99, 100] Alb-uPA mice had to be backcrossed on an immune-deficient background, such as the severe combined immune deficiency (SCID), in order to enable xenotransplantation. Constitutive expression of uPA in the liver induces injury that allows the selective expansion of the transplanted mouse or human hepatocytes.[99]
Another example is the triple knockout (KO) mouse for fumarylacetoacetate hydrolase (*Fah*), recombination activating gene 2 (*Rag2*) and interleukin 2 receptor, gamma chain (*Il2rg*), defined as FRG-KO. FRG-KO mice can be efficiently repopulated with human hepatocytes, as such or on the nonobese diabetic (NOD)-strain background (FRGN).[99, 101, 102] *Rag2* and *Il2rg* are involved in the development of B and T cells, and natural killer (NK) cells respectively. Their KO thus results in impairment of the adaptive immune system.[99, 101, 102] Moreover, backcrossing with the NOD background improves the process of engraftment process and the efficiency of the humanization, as the signal-regulatory protein alpha (*Sirpa*) polymorphism contained in the NOD background hinders phagocytosis and production of inflammatory cytokines in monocyte-derived macrophages.[102-104] When *Fah* is missing, native hepatocytes are continuously damaged by high levels of tyrosine and oxidative stress. Prior to transplantation, FRG(N) mice are therefore treated with nitisinone (or NTBC), which

is also used in clinical practice to block the upstream tyrosine metabolism and prevent liver damage. After transplantation, NTBC is withdrawn to allow selection of the transplanted hepatocytes.

The liver-humanized FRG(N) mouse model has been characterized to a certain extent in regard to lipoprotein and BA metabolism, and hepatic zonation and physiology.[86, 105-108] After transplantation with human hepatocytes, FRG(N) mice display increased LDL and VLDL fractions and a lower HDL fraction compared with wildtype mice, thus significantly shifting the ratio of LDL-cholesterol to HDL-cholesterol towards a more human-like profile.[86] The BA profile in bile revealed a human-like pattern as well, with higher ratio of DCA over beta-MCA, the latter being an exclusive mouse BA.[86, 87, 107] However, a more thorough characterization of the model is still required to compare their translatability to human pathophysiology.

An extensive comparison among the different LHM models has not been performed yet, but each system is likely to present unique advantages or disadvantages for engraftment efficiency and downstream applications.[99] Nevertheless, LHM are being more commonly used for a number of applications, including metabolic and physiological studies, personalized medicine, infectious/viral disease, gene therapy, stem cell biology, pharmacological and disease modeling.[86, 99, 105, 108-112]

1.3 EMERGING *IN VITRO*/*EX VIVO* MODELS TO STUDY CARDIOMETABOLIC DISEASES

Preclinical research also utilizes less challenging *in vitro*/*ex vivo* models, useful to acquire insights and test hypotheses. Furthermore, some of these models can also be used to evaluate molecule properties, providing more relevant information compared with the classical clinical biomarkers that solely quantify the molecule absolute levels.

1.3.1 Improved human hepatocyte-like cellular systems

Primary human hepatocytes (PHH) are regarded as the "gold standard" to study *in vitro* hepatic lipid metabolism and hepatic steatosis/NAFLD. However, these cells present major drawbacks such as short lifespan, substantial inter-donor differences, inability to proliferate, and loss of transporters and metabolizing enzymes.[113] It has also been shown that there is a rapid de-differentiation that occurs in PHH cultured under standard monolayer conditions (2D), which also affects lipid metabolism.[114, 115] Various systems of 3D and/or microfluidic culture have been developed to improve the morphology and functionality of PHH.[116, 117] In parallel, systems with human hepatocyte-like cell lines easier to handle and relatively less expensive are being developed.

1.3.1.1 Culturing with human serum

Several human hepatocyte-like immortalized cell lines (*e.g.*, HepG2, Huh7 and Huh7.5) have been generated, but none of them completely mimic human hepatocytes *in vivo* under standard culturing conditions, *i.e.*, using culturing media supplemented with fetal bovine serum (FBS). For instance, HepG2 cells cultured under standard conditions secrete aberrant

lipoproteins and display HDL-sized APOB-containing lipoproteins in the cell medium.[113] Culturing media supplemented with 2% human serum (HS) instead of 10% FBS considerably improve the usefulness of HepG2 cells to study lipid metabolism.[113, 118] HepG2 cells cultured with HS display APOB-containing LDL-sized and APOA1-containing HDL-sized particles, higher TGs, BAs and PCSK9 levels in the cell medium, and higher beta-oxidation and insulin sensitivity.[113, 118] These changes are probably secondary to an increased expression of genes involved in differentiation and lipid metabolism.[113] Thus, this translational model can be used in studies of hepatic metabolism and pathology, although a direct functional comparison with PHH is required.

1.3.1.2 Cell genome editing and the CRISPR technology

Human hepatocyte-like cell lines exhibit multiple chromosomal aberrations that affect gene expression and metabolism with respect to hepatocytes *in vivo*.[119-122] For instance, two isoforms of *SOAT* catalyze the formation of intracellular CEs from fatty acids and cholesterol: *SOAT1*, ubiquitously expressed, and *SOAT2*, which is solely expressed in hepatocytes and enterocytes.[123] In contrast to *SOAT1*, *SOAT2* determines the amount of CEs secreted in APOB-containing lipoproteins, and thereby contributes to the CEs in the circulation.[124] Moreover, *SOAT2* regulates the intracellular levels of CEs and TGs.[125, 126] Conversely to normal hepatocytes *in vivo* which only express *SOAT2*, both isoforms are expressed in HepG2, Huh7.5 and also in PHH. Hence, KO or editing of genes that alter the metabolism of hepatocyte-like cells can improve their phenotype with respect to the *in vivo* situation.

Since the discovery of the DNA double helix, the possibility of creating site-specific changes to the genome of cells and organisms has been contemplating. In the latest years, the genome engineering field has profoundly transformed thanks to the clustered regularly interspaced short palindromic repeats (CRISPR) technology (for the history of CRISPR development see [127]). The simplicity and versatility of RNA-guided CRISPR programming, together with the specific DNA cleaving mechanism, have enabled remarkable developments in genome engineering, allowing laboratories around the world to edit genomes of a wide range of cells and organisms.[127]

The CRISPR technology is based on base-pairing rules between an engineered RNA and the target DNA locus. It requires a single guide RNA (gRNA) together with the CRISPR-associated protein (Cas) enzyme to introduce a site-specific double-stranded break in the DNA. The engineered gRNA is characterized by a 20-nucleotide sequence at the 5' side that determines binding to the DNA target. The Cas9-mediated cleavage occurs only when a PAM (protospacer adjacent motif) signature sequence of 2-6 nucleotides follows the sequence of interest on the DNA. The RNA-guided nuclease function is reconstituted in mammalian cells through the heterologous expression of RNA components together with Cas9.[128] After the DNA break, the genome locus becomes substrate for the endogenous cellular DNA repair machinery that catalyzes the error-prone non-homologous end joining (NHEJ) or the high-fidelity homology-directed repair (HDR). NHEJ gives rise to small insertion/deletion (indels) that disrupt the translational reading frame of a coding sequence

leading to premature stop codon and ideally a gene KO. Alternately, HDR can be used to insert desired sequences or to introduce specific point mutations through recombination of the target DNA site with exogenously-supplied DNA templates.[129]
A main concern regarding the CRISPR-Cas9-mediated genome editing is the off-target events that potentially lead to disruption of other DNA sites than the desired one. A strategy combining a mutant nickase version of Cas9 with a pair of gRNAs complementary to opposite strands of the target DNA site has been developed in order to improve the specificity. This combination allows the DNA break to occur only when both guides induce single-stranded nicks at the target region, generating higher specificity and minimizing off-targets.[130] An example of CRISPR strategy is briefly described in 3.2.1.

1.3.2 Human aortic proteoglycan-binding assay

High levels of APOB-containing lipoproteins are the most common risk factor for ASCVD and CMD,[4] and their atherogenicity is explained by the ability to bind to the arterial PG.[5, 10] Particle retention within the arterial wall is indeed the necessary step to their further modifications leading to the development of atherosclerosis.[131, 132] PG are formed by a protein core attached to negatively-charged polysaccharide glycosaminoglycans, which can interact with specific positively-charged residues in APOB.[10, 131] The composition and size of APOB-containing lipoproteins (together with the composition and interaction with extracellular PG) determine the binding and thus the atherogenicity of the particle.[10, 131, 132] Both APOB100 and APOB48 can bind PG, although different sites are involved.[131, 132] Furthermore, other APOs such as APOE show PG-binding sites, which can explain the atherogenicity of entrapped APOE-rich remnants, and even mature HDLs.[131, 132] It is possible to measure this lipoprotein property using PG isolated from human aorta, as described in 3.2.5.5.

1.3.3 Cholesterol efflux capacity-cellular models

The increase of plasma HDL-cholesterol levels has been proposed as a potential therapeutic strategy to prevent ASCVD and CMD. However, previous GWAS and clinical studies show contrasting results.[4, 8, 13, 133-135] Thus, as alternative to plasma HDL-cholesterol levels, the notion of "HDL functionality" has emerged as the key-determinant for the HDL-mediated protection from ASCVD.[8] One of the most studied HDL functionality is CEC, *i.e.*, the transfer of FC from the intracellular compartment towards an extracellular acceptor, such as HDLs.[136] It represents one of the initial and most important steps of RCT transport, a process by which cholesterol is transferred from peripheral tissues to the liver for subsequent elimination in the feces.[137] In humans, HDL-CEC has been negatively correlated with both intima media thickness and coronary artery disease.[138] The composition of HDL subclasses appears to be the major determinant of serum CEC,[139] which occurs by four mechanisms:[140] active transport mediated by (a) ABCA1 to APOA1 and pre-beta HDLs, and by (b) ABCG1 to pre-beta and mature HDLs, (c) SRB1-mediated bidirectional efflux to mature HDLs, and (d) aqueous diffusion to mature HDLs.
Several cellular models are used to measure CEC, although there is no common consensus about the experimental procedure.[141, 142] In general, cells are incubated with labeled

cholesterol, equilibrated to distribute the cholesterol equally among the cell pools, treated with various compounds (if necessary) and finally incubated with a cholesterol acceptor (*e.g.*, serum or isolated lipoproteins) to assess its efflux capacity.

Aqueous diffusion is a passive transport mechanism driven by a cholesterol concentration gradient at the cell membrane. Every cell type is able to passively efflux FC, and murine immortalized macrophages such as J774A.1 and RAW 264.7 are most commonly used to measure the aqueous diffusion-CEC. When these cells are stimulated with cAMP, *Abca1* is up-regulated, thus allowing to assess the efflux via ABCA1.[143] For the ABCG1 pathway, common mammalian lines (such as Chinese hamster ovary cells) overexpressing ABCG1 have been used, although both the model and the role of this transporter in RCT transport are still debated.[144-149] Finally, the cell line Fu5AH (rat hepatoma), is commonly used to estimate the SRB1-mediated CEC.[139, 150] Immunoblot analysis has indeed shown that Fu5AH cells express high levels of SRB1.[151]

2 AIM AND SIGNIFICANCE

The overall aim of this thesis was to develop and characterize new experimental models relevant to the human condition in respect to liver lipid and lipoprotein metabolism, and to use these models to validate new therapeutic targets to treat CMD.
The specific aims in the individual papers were:

Papers I and **II**
To characterize the lipoprotein and liver lipid metabolism in LHM, and the effects of HFHSD. Specifically, we focused on assessing liver-related physiological and pharmacological species-specific differences between humans and mice, and the variability of response among LHM repopulated with hepatocytes from different human donors.
We also investigated the development of diet-induced CMD in LHM fed a high-fat/high-fructose/high-cholesterol diet (NASH-diet), and presented these data as preliminary results not included in the constituent papers.

Paper III
To generate *SOAT2*-only-HepG2 and *SOAT2*-only-Huh7.5 hepatocyte-like-cell lines by KO of *SOAT1* using the CRISPR technology, and to assess the phenotypical changes in lipid metabolism when culturing these cells with either FBS or HS.

Paper IV
To generate a *CIDEC*-KO-HepG2 cell model using the CRISPR technology to be used to investigate the effects of *CIDEC* inhibition on human hepatic lipid and carbohydrate metabolism, and its potential role as therapeutic target for hepatic steatosis.

3 METHODOLOGY

The results presented in this thesis have been generated using advanced animal models and by establishing unique cellular models. Basic and advanced molecular techniques were used together with computational biology analyses. A more comprehensive description for every experimental procedure can be found in the papers constituting the thesis.

3.1 EXPERIMENTAL MODELS

3.1.1 Liver-humanized mice and dietary regimens

Mice knocked out for *Fah*, *Rag2* and *Il2rg* (FRG-KO) on the NOD background (FRGN) were engrafted with hepatocytes as previously described.[101, 102, 104] LHM were transplanted with human hepatocytes from different donors, as described in detail in **Papers I** and **II**. Liver-murinized mice (LMM) are FRGN mice engrafted with NOD mouse hepatocytes and served as mouse reference. Both LMM and LHM were fed a maintenance diet (**Paper I**), HFHSD for eight weeks (**Paper II**), or NASH-diet for eight or twelve weeks. Details on the diet composition are provided in Table 3.1. LHM were also treated with the LXR agonist GW3965 (30 mg/kg/day) in **Paper I**.

	Maintenance diet (Paper I)	HFHSD (Paper II)	NASH-diet
Fat	**11.1**	**24.0**	**21.9**
Cholesterol	< 0.01	0.02	2.0
SFA	3.7	7.0	8.7
MUFA	4.0	7.9	6.7
PUFA	2.3	7.3	3.8
Carbohydrate	**51.8**	**41.0**	**44.9**
Starch	32.6	20.1	11.1
Glucose	0.1	0.0	0.0
Fructose	0.1	0.0	22.1
Sucrose	0.9	20.1	10.6
Protein	**18.9**	**24.0**	**22.5**

Table 3.1. Formula composition of maintenance diet, high-fat/high-sucrose diet (HFHSD), and high-fat/high-fructose/high-cholesterol diet (NASH-diet). *Values indicate the approximate weight percentages. Abbreviations: SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids.*

3.1.2 Human hepatocyte-like cells and culturing conditions

HepG2 (ATCC) and Huh7.5 cell lines (a kind gift from Prof. Charles M. Rice, The Rockefeller University, NY, USA) were cultured at 37 °C and 5% CO_2 in Dulbecco Modified Eagle Medium (DMEM) supplemented with either 10% FBS or 2% HS, 100 U/mL penicillin, and 100 U/mL streptomycin (Thermo Fisher Scientific). The method to culture human hepatocyte-like cells with HS was previously described.[113] The generated *SOAT2*-only-HepG2 and *SOAT2*-only-Huh7.5 cells were cultured in media supplemented with either FBS or HS (**Paper III)**, whereas the *CIDEC*-KO-HepG2 cells were cultured in medium supplemented with FBS (**Paper IV**).

3.2 EXPERIMENTAL PROCEDURES

3.2.1 CRISPR strategy for cell genome editing

SOAT2-only-HepG2 and *SOAT2*-only-Huh7.5 cells were generated by KO of the *SOAT1* gene (**Paper III**), and *CIDEC*-KO-HepG2 cells were generated by KO of the *CIDEC* gene (**Paper IV**) following the guidelines provided by Feng Zhang lab.[128] In both strategies, custom gRNAs for each target were designed *in silico*. gRNA sequences were cloned into an expression plasmid bearing both single gRNA scaffold backbone and Cas9, the endonuclease system cleaving DNA. To minimize potential genome off-targets editing, two gRNAs and Cas9 nickase were used.[130] Transfected cells were then clonally expanded to obtain cell lines bearing homogenous mutations in the defined editing sites. Successful biallelic out-of-frame editing was detected by Sanger sequencing at KIGene, Karolinska Institutet (Stockholm, Sweden). *SOAT1* or *CIDEC* mRNA, protein, or activity levels were assessed in order to confirm the functional KO in the edited cells.

3.2.2 RNA extraction, cDNA synthesis and quantitative real-time PCR

Total RNA was extracted from snap-frozen organs (**Paper I**) or cells (**Papers III** and **IV**) using commercially available spin columns with silica membranes or a phenol-guanidine isothiocyanate-based solution. RNA was reverse-transcribed to cDNA with specific kits. Quantitative real-time polymerase chain reaction (qPCR) was performed with Fast SYBR Green or TaqMan Universal PCR Master Mixes (Thermo Fisher Scientific). The primers used in LHM samples were specific for human or mouse orthologous genes (**Paper I**). Arbitrary units were calculated by linearization of the C_t values and normalized to human and mouse RNA18S5/Rn18s (18S) rRNA for LHM organs (**Paper I**) or glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) for human hepatocyte-like cells (**Papers III** and **IV**).

3.2.3 Western blot

Whole-cell homogenates were prepared from snap-frozen livers (**Paper I**) or cells (**Paper III**). Equal amounts of protein from individual samples in each group were pooled. To perform a titration analysis for the LDLR, different amounts of protein underwent gel electrophoresis. Subsequently, proteins were transferred to nitrocellulose membrane and blocked before incubation with an antibody against LDLR. Odyssey Fc (LI-COR

Biosciences) was used to visualize and quantify the bands. LDLR protein expression was quantified by calculation of the first derivative of the linear regression function interpolating the titration points for each group pool.

3.2.4 Enzymatic activity assays

Plasma lipid transfer activities of CETP and PLTP were assessed in the sera of LMM and LHM with fluorometric assays in kinetic measurement (**Paper I**). SOAT enzymatic activities were assessed in microsomes from unedited and *SOAT2*-only-HepG2 and *SOAT2*-only-Huh7.5 cells (**Paper III**) as previously described.[152]

3.2.5 Analysis of lipoprotein metabolism

3.2.5.1 Quantification of lipoprotein lipids

Lipoproteins from serum/plasma (**Papers I** and **II**) or cell medium (**Paper III**) were separated by size-exclusion chromatography (SEC), and lipids were quantified by real-time detection system as previously described.[113, 141, 153]

3.2.5.2 Quantification of mediators of lipoprotein metabolism

Commercially available enzyme-linked immunosorbent assay (ELISA) kits were used to assess the levels of CETP, Lp(a) and PCSK9 in serum (**Paper I**), according to the manufacturer's protocol. APOB, Lp(a) and PCSK9 levels were also assessed in cell medium (**Paper III**).

3.2.5.3 Apolipoprotein B mRNA editing assay

To quantify the editing of APOB mRNA in the chimeric livers of LHM (**Paper I**), we developed a qPCR-based assay in collaboration with TATAA Biocenter (Gothenburg, Sweden). Two outer primer pairs were designed to specifically amplify APOB in either human or mouse cDNA by PCR. Amplicons (or cDNA as such) were analyzed by qPCR with inner degenerated primers amplifying both APOB48 and APOB100 transcripts, and two probes distinguishing between the two transcripts. A standard curve was established using two gBlocks bearing APOB48 and APOB100 variants, respectively. The duplex assay with both outer and inner degenerated primers was used to distinguish the editing in either the human or mouse component, whereas the inner assay alone with cDNA was used to measure the overall sample editing.

3.2.5.4 Apolipoprotein composition in isolated lipoproteins

In **Paper I**, sequential differential micro-ultra-centrifugation in deuterium oxide (D_2O)/sucrose was used to separate serum lipoproteins, as previously described.[154] Lipoprotein fractions underwent gel electrophoresis to separate APOs and lipoprotein-associated proteins. Gels were stained with Coomassie G-250 and protein bands were identified based on fraction localization and molecular weight.

3.2.5.5 Binding to human aortic proteoglycans

Solid-phase assay was used in **Paper I** to test serum lipoprotein binding to human aortic PG (haPG) isolated from intima-media of human aortas.[10, 155, 156] After incubation with haPG, the amount of bound cholesterol was quantified and reflects the affinity of the sample for haPG. The assay has been standardized to predict the atherogenic potential of serum/plasma or isolated lipoproteins.

3.2.5.6 Cholesterol efflux capacity

In **Paper I**, the whole-serum CEC of LHM treated with or without LXR agonist was assessed with a radiolabeled cellular assay as previously described.[141] J774A.1 cells (ATCC) were cultured at 37 °C and 5% CO_2 in Roswell Park Memorial Institute (RPMI) 1640 medium containing 10% FBS and 50 μg/mL gentamicin (Thermo Fisher Scientific). After plating, cells were incubated with [1,2-3H(N)]-cholesterol and an unspecific SOAT inhibitor, equilibrated with bovine serum albumin (BSA) to distribute the labelled cholesterol equally among the cell pools, and treated with either 8-(4-chlorophenylthio)-cAMP (cpt-cAMP) or vehicle. Finally, cells were incubated with or without serum acceptor. Cells incubated without acceptor served as baseline for radiolabeled cholesterol. Radioactivity was determined in cell lysates or medium by liquid scintillation. The percentage of cholesterol efflux was calculated as the ratio of radioactivity in the medium to baseline radioactivity. Aqueous diffusion was evaluated by J774A.1 cells cultured under basal conditions, whereas the efflux mediated via ABCA1 was the difference between the cholesterol effluxes measured by J774A.1 incubated with cpt-cAMP and under basal conditions.

3.2.6 Analysis of intracellular lipid metabolism

3.2.6.1 Quantification of intracellular lipids

Lipids from livers (**Papers I** and **II**), cells (**Papers III** and **IV**) or portions of the descending aorta from LMM and LHM fed a maintenance or NASH-diet were extracted with chloroform/methanol 2:1 by volume (also known as Folch extraction) as previously described.[113, 157] Parts of the extracts were mixed with 1% TritonX-100 and lipids were solubilized in water after evaporation of organic solvents. TGs were quantified by a colorimetric enzymatic method on the water extracts. In **Papers I** and **II**, total cholesterol (TC), FC, lathosterol, and lanosterol were analyzed from liver Folch extracts by isotope dilution GC–MS.[158, 159] Lathosterol and lanosterol are used as biomarkers of cholesterol synthesis.[160] TC and FC were only quantified in the descending aortas. In **Papers III** and **IV**, colorimetric enzymatic methods on the water extracts were used to quantify TC and FC. CE mass was calculated by subtracting FC from TC and multiplying by 1.67, a conversion factor considering the higher molar mass of CEs compared with FC.

3.2.6.2 Analysis of liver and biliary bile acids

7alpha-hydroxy-4-cholesten-3-one (C4) is used as a biomarker of BA synthesis, and was quantified in liver Folch extracts (**Papers I** and **II)** by isotope dilution LC-MS/MS.[161] BAs

were quantified in livers by LC-MS/MS after extraction with Bile Acids Kit (Biocrates Life Sciences)[162] (**Paper I**), and in gallbladders by another LC-MS/MS-based method[86, 163] (**Paper II**).

3.2.6.3 Histological analyses

Sections of 4 μm were cut on a rotary microtome from paraffin-embedded livers and stained with hematoxylin and eosin (H&E) (**Paper I**). Sections of optimal cutting temperature (OCT) compound-embedded hearts from LMM and LHM fed a maintenance or NASH-diet were stained with H&E, Oil Red O (ORO), or anti-mouse cluster of differentiation (CD) 68 antibody conjugate. Sections were visualized with bright-field microscopy.

3.2.7 RNA sequencing and transcriptomic analysis

3.2.7.1 Preparation and sequencing of RNA libraries

In **Papers I** and **II**, RNA was extracted from snap-frozen livers and libraries were prepared according to the Illumina TruSeq Stranded mRNA Sample Preparation protocol (Illumina). All samples showed RNA integrity number (RIN) values above 9.0, and poly(A) selection was used to isolate mRNA. After fragmentation, the cDNA strands were synthetized and modified to facilitate strand hybridization. DNA products were purified and enriched by PCR. RNA libraries were validated using the Bioanalyzer Agilent DNA 1000 (Agilent Technologies), pooled and sequenced by Illumina NovaSeq 6000, PE 2x50bp system.

3.2.7.2 Mapping to human or mouse genome

The RNA sequencing (RNA-seq) reads were trimmed, filtered and mapped by STAR mapper[164] to the human genome version GRCh38 and to the mouse genome version GRCm38 obtained from Ensembl release 96,[165] together with gene annotations (**Papers I** and **II**). In **Paper II**, orthologous genes were identified querying Ensembl with *biomaRt*.[166] Gene expression was then quantified for each sample (**Papers I** and **II**). Principal component analysis (PCA) revealed the absence of outliers.

3.2.7.3 Differential expression analysis

DESeq2[167] in R 3.5.1 was used to assess differentially expressed genes (**Papers I** and **II**). Logarithmic-fold change was shrank using the Approximate Posterior Estimation for global linear models (apeglm) method to improve the estimate of the effect size.[168] Wald's test was performed to compare the gene expression in liver samples. In all comparisons, *p*-values were adjusted for multiple testing using the Benjamini-Hochberg method.[169]

3.2.7.4 Pathway and Gene Ontology analysis

Gene Ontology (GO) enrichment pathway analyses were performed using the R library *gage*[170] (**Paper I**) or the enrichGO function of the R library *clusterProfiler*[171] selecting the Biological Processes ontology (**Paper II**).

3.3 STATISTICAL ANALYSIS

In **Papers I** and **II**, the robust regression and outlier removal (ROUT) method (Q = 5%) was performed prospectively to remove group outliers. Data from pooled samples and lipoprotein chromatograms are expressed as mean, ± standard error of the mean (SEM) when indicated. Spearman rank correlation analysis (**Paper I**) or Pearson correlation coefficient (**Paper II**) were used to determine associations between continuous parameters. Statistical significance was set to $\alpha < 0.05$, unless otherwise stated. All statistical analyses and graphs were performed using Statistica (TIBCO Software Inc.) and GraphPad Prism (GraphPad Software, Inc.). For transcriptomic analysis, see 3.2.7.

In **Paper I**, a nonparametric analysis was used. Data are expressed as medians and interquartile range or scattered dots. Significant differences among groups were tested using Kruskal-Wallis test followed by Dunn's multiple comparison test. After Holm-Bonferroni adjustment, statistical significance was set at the level of $\alpha < 0.01$ ($\alpha < 0.05$ for *post hoc* analyses). In the LXR study the α-value was kept to 0.05, and Mann-Whitney U test was used to compare two groups.

In **Paper II**, all data were analyzed after log-transformation to reduce intragroup variability. Normality was tested for all variables by Shapiro-Wilk W test before comparing the groups using a parametric analysis. Data are presented as means of scattered dots or ± SEM. One-way analysis of variance (ANOVA) followed by *post hoc* comparison using Tukey honestly significant difference (HSD) test were used for contrast among the groups.

In **Papers III** and **IV**, data from individual wells or flasks are expressed as means ± SEM or scattered dots. One-way ANOVA and Tukey HSD *post hoc* test were used to compare multiple groups. Significant differences between two groups were tested by unpaired two-tailed *t* test.

Data from LMM and LHM fed a maintenance or NASH-diet for eight or twelve weeks are presented as scattered dots with means represented by bars. To assess the effects of liver humanization, dietary regimen and dietary feeding time, significant differences were tested by factorial ANOVA. Data were log-transformed to stabilize variances.

3.4 ETHICAL CONSIDERATIONS

The correctness of research with animal models and humans is controlled by ethical review committees, which set the principles of good research practice considering its wider consequences. The animal studies presented in this thesis were approved by the appropriate ethical authority as indicated: DN000024; 2010/678-31/3; S82-13; 2017/269-31; LREC 01-016. Research efforts worldwide strive to gain a better understanding of the pathophysiological mechanisms underlying several morbidities in order to prevent disease diffusion, discover new therapies, and to ultimately improve the quality of our lives. Before new discoveries are tested in humans, various models in preclinical and clinical research settings are used to assess research hypotheses and perform toxicology studies, such as human-derived cells or tissues and animals. However, the use of these models is known to represent an ethical dilemma for multiple reasons, including their relevance to the human condition, an aspect that unfortunately seems to be largely underestimated.

3.4.1 Reflections from Papers I and II

The studies with LHM allowed to deepen the considerations on the human translatability of animal models in preclinical research. Moreover, because LHM receive cells from human donors, they also entail important reflections on the possibility to perform personalized studies and on the protection of the personal data gained.

Extensive mechanistic studies on multifactorial diseases involving several organs (such as CMD) cannot be performed in regular cellular models due to major limitations, nor in humans unless proven hypotheses are obtained in animal models. However, differences among the species limit the translatability of animal models to the human condition, as widely discussed. LHM were chosen as the central model within the EU-FP7 project HUMAN (Health and the Understanding of Metabolism, Aging and Nutrition), which aimed to study the effects of genetic and dietary factors involved in the development of human CMD in humanized mouse models. The validation of preclinical models with improved human relevance provides the possibility to dramatically reduce the number of animals used in research. This is in accordance with the principle of the 3Rs, which represents a framework for conducting high-quality research with the aim to develop alternative approaches to avoid the animal use: Replace (*in vitro* using cellular models), Refine (considerations on animal welfare) and Reduce (use the minimal number of animals sufficient for statistical analysis). As also indicated within the HUMAN research project, "There must be a balance between the objectives of the research and the means used."

The PHH used to repopulate LHM can be selected from cryopreserved biobanks based on genotype, age and clinical/metabolic data. Because LHM can reproduce traits of the original phenotype,[108-110] human donors can theoretically be recalled for additional testing based on the data obtained from LHM. This potential application of personalized medicine represents another ethical justification for the use of humanized animal models.

Moreover, all partners within HUMAN had to follow international guidelines and ethical rules for human samples (Declaration of Helsinki), information about the donors and animal experimentation. In fact, the project involved patients, healthy volunteers, human genetic and biological samples, and collection of data where subjects are identified with a study-ID number. The processing of this sensitive information (*e.g.*, health status or ethnicity) had to be carried out in accordance with the General Data Protection Regulation (GDPR) and thus preventing tracking by external parties.

3.4.2 Reflections from Papers III and IV

If the intrinsic species differences of the animal models represent a major limitation, the use of cellular models entails likewise problems for their clinical translatability. In fact, immortalized cell lines derive from tissues and cells carrying severe diseases, and their genetic instability affects their metabolism with respect to the original tissue. Similarly, primary cells can easily undergo phenotype changes once removed from the original tissue, and their culturing is cumbersome due to limited proliferative capacity and short lifespan. Hence, the development of immortalized cellular models better representing the physiological situation of cells *in vivo* is a necessary step to improve the human relevance of *in vitro*

models for preclinical studies, and also to reduce the number of animal studies.
HepG2 and Huh7.5 are human hepatocyte-like immortalized cells derived from human hepatoblastoma and hepatocellular carcinoma, respectively.[172, 173] Such cell lines can proliferate indefinitely and are routinely used in preclinical research. Moreover, they do not require an ethical approval, as they do not present neither developmental potency nor possibility to cross-contaminate human subjects. Although the use of genome-editing techniques such as CRISPR raises strong ethical controversy for their potential application in humans and human embryonic stem cells, genome editing is allowed in cells lines, and can be used to KO genes that impair their phenotype. This possibility represents a great opportunity to improve cellular models. In addition, culturing human hepatocyte-like cells in media supplemented with 2% HS was shown to greatly improve their functionality and usefulness in metabolic studies.[113] Altogether, these improvements provide a better model to study *in vivo* hepatocyte metabolism. Of note, the use of human blood (commercially available) does not require ethical permission.

4 RESULTS AND DISCUSSION

4.1 A HUMANIZED LIPOPROTEIN AND LIVER METABOLISM IN LIVER-HUMANIZED MICE

4.1.1 Liver-humanized mice exhibit a human-like lipoprotein profile similar to the human donor

The main part of the cholesterol transported in the bloodstream is carried in LDL particles in humans and in HDL particles in mice. LHM were shown to display a typical human-like distribution of plasma cholesterol, shifting the cholesterol transport into LDL rather than HDL particles.[86] In **Papers I** and **II**, we widened this observation by finding that the humanization of the cholesterol lipoprotein profile depends on the hepatocytes of the human donor. As shown in Figure 4.1, distinct and donor-specific cholesterol lipoprotein profiles were consistently found in various groups of LHM engrafted with human hepatocytes from different donors. The intriguing idea that LHM can recapitulate the metabolic features of the human donor has already been proposed in previous studies.[108-110] Further insight came from the comparison of the lipoprotein profiles of the human donor A with those of the LHM engrafted with the PHH from this donor (LHM-A) and LMM (Figure 4.1A). Human donor A and LHM-A displayed similar – but not identical – cholesterol lipoprotein profiles, conversely to the typical mouse distribution found in LMM. Likely, the minimal residual activity of the mouse liver, the interaction between the tissues of the mouse recipient and the human donor, and the mouse peripheral metabolism prevent the achievement of identical lipoprotein profiles.

4.1.2 Circulating APOB100 and hepatic LDLR (but not CETP) determine the humanization of the lipoprotein profile

In **Paper I**, we extended the investigation on the species-specific differences between human and mice in liver metabolism responsible for the humanization of the lipoprotein profile. Among these (see 1.1.4), CETP is a plasma lipid transfer protein expressed in humans but not in mice. CETP promotes the equimolar exchange of CEs contained in HDLs for TGs from VLDL and LDL particles. Thus, its presence in humans is considered one of the main mechanisms in determining the higher amount of plasma cholesterol carried in APOB-containing lipoproteins.[81, 82] Humans express CETP predominantly in the spleen, adipose tissue and liver, hence we sought to assess whether LHM expressed CETP in the liver. We analyzed the CETP expression at mRNA and protein level in the liver, and CETP protein and activity in the circulation, but we were unable to detect it neither in the liver nor in the blood (**Paper I**). The hepatic expression of CETP in humans is driven by macrophage-resident Kupffer cells.[174] This explains the absence of CETP in LHM, which are only engrafted with human hepatocytes and, consequently, have resident mouse Kupffer cells in the chimeric livers. Therefore, the humanization of the lipoprotein profile in LHM is not dependent of CETP. In humans, *CETP* homozygous loss-of-function mutations are often associated with hyperalphalipoproteinemia, *i.e.*, an excess of HDL- over LDL-cholesterol.[175, 176] This

suggests that CETP in humans plays an extremely important role in regulating the homeostasis of plasma cholesterol, and that further physiological mechanisms underlie the different distribution of plasma cholesterol in humans and mice.

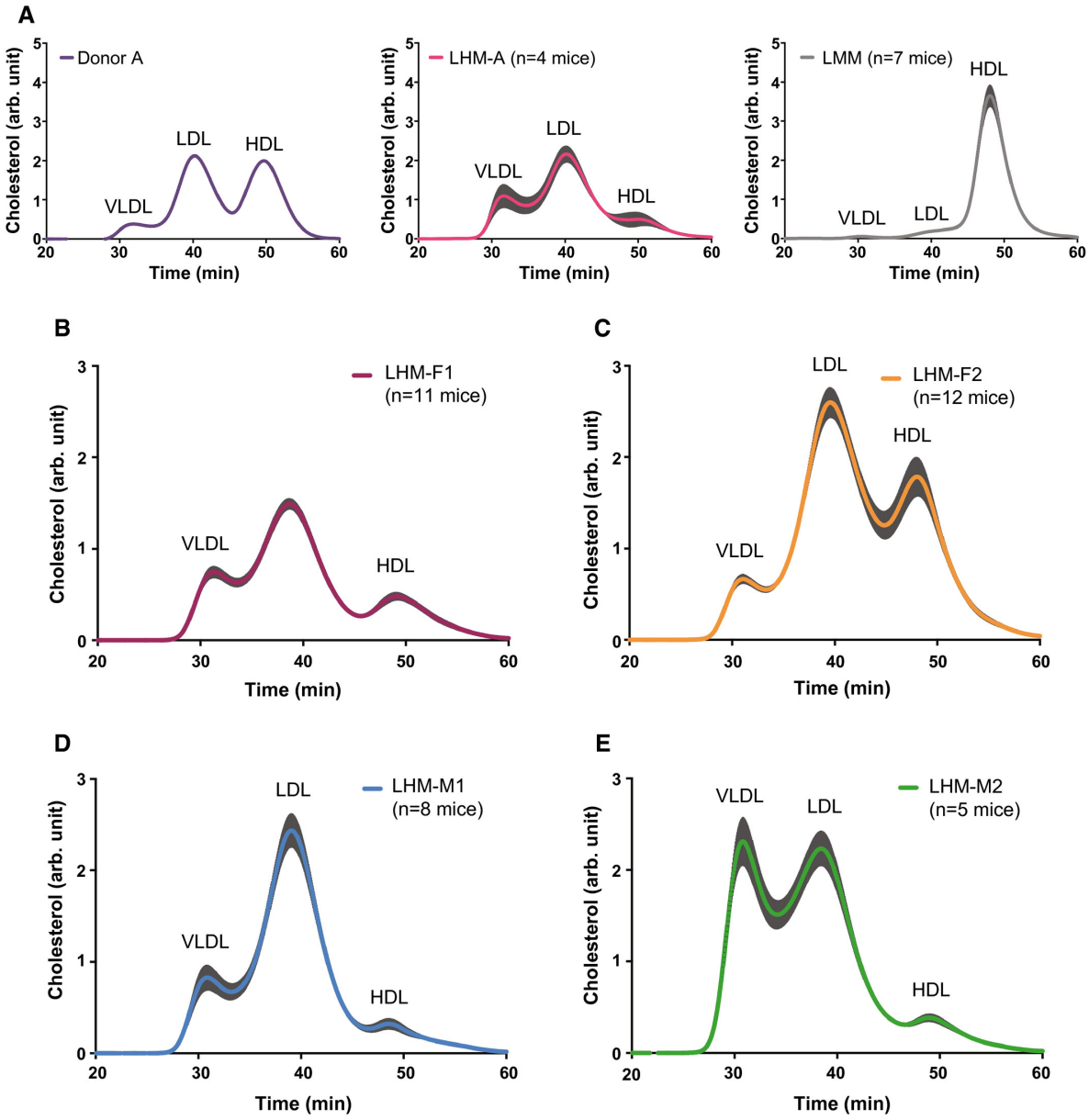

Figure 4.1 The human-like cholesterol lipoprotein profile in LHM depends on the hepatocytes of the human donor. *Lipoproteins were separated by SEC and cholesterol was quantified. (**A**) Cholesterol profile of human donor A, of the LHM engrafted with the PHH from this donor (LHM-A), and of LMM. Cholesterol profiles of LHM transplanted with F1 (**B**), F2 (**C**), M1 (**D**), or M2 (**E**) hepatocytes. Profiles represent the mean chromatograms, and the shaded area around them represents the SEM. From **Paper I**.*

We then hypothesized that the predominant distribution of cholesterol in LDL particles resulted from higher levels of circulating APOB100, as lipoproteins containing APOB100 rather than APOB48 have higher half-life in the circulation.[177] Among other interspecies differences in liver and lipoprotein metabolism, humans do not edit APOB mRNA in the

liver, thereby producing only APOB100.[80] Moreover, humans have lower hepatic LDL clearance compared with mice.[45] As shown in Figures 4.2A and 4.2C respectively, the editing of APOB mRNA and the expression of LDLR in the liver of LHM were approximately 70% lower compared with LMM. Furthermore, LHM exhibited higher levels of PCSK9 (Figure 4.2D), a posttranslational negative regulator of LDLR in the circulation.[4] Altogether, these changes synergistically increase the levels of APOB100 in the circulation of LHM (Figure 4.2B), thereby conferring increased half-life on VLDLs and LDLs.[177] Finally, the lipoproteins of LHM displayed binding to haPG and plasma lipid levels similar to normal physiological values in humans (**Paper I**). These results corroborate LHM as a clinically translatable model for studies of lipoprotein metabolism and atherosclerosis.

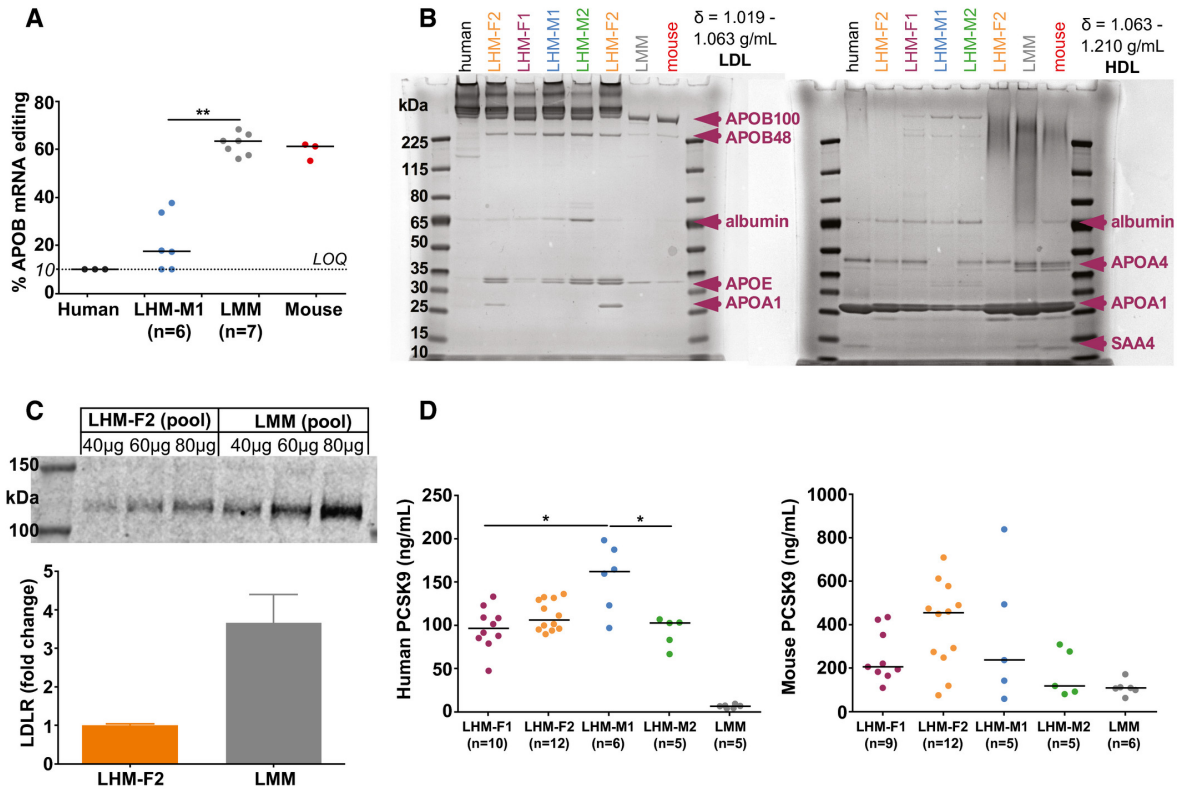

Figure 4.2. Higher levels of APOB100 in the circulation and lower LDLR expression in the liver determine the humanization of the lipoprotein profile in LHM. *(**A**) APOB mRNA editing quantified in liver by a qPCR–based assay. Lines indicate the medians of scattered dots. Mann-Whitney U Test; ** $p < 0.01$. (**B**) APO composition of LDLs and HDLs. Serum lipoproteins were isolated and underwent gel electrophoresis before staining. (**C**) LDLR protein expression in the liver by Western blot. Data are presented as mean and SEM. (**D**) Human and mouse PCSK9 in the circulation quantified by ELISA. Lines indicate the medians of scattered dots. Kruskal-Wallis test followed by multiple comparisons; * $p < 0.05$. Abbreviations: SAA, serum amyloid A. From **Paper I**.*

4.1.3 Liver-humanized mice are resistant to a high-fat/high-sucrose diet

Conversely to conventional mouse models, LHM fed a maintenance diet appeared to be a translatable model to study human hepatic and lipoprotein metabolism (**Paper I**). Therefore, in **Paper II** we aimed to test whether the feeding of LHM a HFHSD, consisting of 24% fat

and 20% sucrose, for eight weeks could impair the lipid phenotype of these mice. We considered eight weeks to be enough to see pathological effects without impairing cell survival and liver inflammation. The analysis of plasma lipids in LHM fed HFHSD confirmed higher levels of cholesterol and TGs in the APOB-containing lipoproteins, but plasma levels of TC did not significantly differ to LMM after HFHSD (**Paper II**). Moreover, plasma levels of TC and TGs were similar between LHM fed a maintenance diet or a HFHSD (**Papers I** and **II**). These data indicate that LHM as well are resistant to the development of hyperlipidemia after lipid and carbohydrate challenge, as seen in several conventional mouse models.

Considering the resistance to HFHSD, we could use the data from **Paper II** to infer further differences in liver lipid metabolism between LMM and LHM, thanks also to the transcriptomic analysis to investigate differences in gene expression underlying the metabolic phenotype. LHM fed a HFHSD exhibited significantly lower levels of HDL-cholesterol compared with LMM, as observed also on the maintenance diet (**Paper I**). In mice, the hepatic expression of *Abca1* is the major determinant for circulating HDLs.[24] Analysis of the liver transcriptome revealed *ABCA1* together with several genes involved in the synthesis, maturation and the structure of HDLs to be downregulated in LHM compared with LMM (**Paper II**). Because the transfer of cholesterol from HDLs to APOB-containing lipoproteins mediated by CETP is absent both in LMM and LHM (**Paper I**), the lower levels of cholesterol circulating in the HDL fraction of LHM seem to stem from the reduced hepatic expression of several HDL-associated genes.

Further confirmation for the resistance of LHM to HFHSD came from the analysis of the liver lipids. Overall, both hepatic TG and TC levels were similar between LMM and LHM (**Paper II**). However, LHM exhibited higher plasma levels of TGs compared with LMM (**Paper II**) The expression of genes associated with TG synthesis, mobilization, and secretion were upregulated, whereas genes important for plasma lipolysis and TG uptake (*e.g.*, *LPL* and *LIPC*/*HL*) were downregulated in the liver of LHM (**Paper II**). These results suggest that an increase in hepatic TG synthesis and secretion, and a decreased lipolysis in plasma seem to underlie the higher levels of plasma TGs in LHM.

Hepatic cholesterol metabolism is discussed more in detail in 4.1.5.

4.1.4 Liver-humanized mice develop atherosclerosis on a NASH-diet

High plasma levels of APOB-containing lipoproteins are a prerequisite for atherogenesis. However, the resistance of LHM to HFHSD hinder their use for studies of CMD. This was seen also in another strain of LHM, that did not develop atherosclerosis after ten weeks of feeding a high-fat diet with 0.3% cholesterol.[178] Hence, we fed LMM and LHM a NASH-diet consisting of 21% fat, 22% fructose and 2% cholesterol for eight or twelve weeks which previously has been shown to induce NASH, insulin resistance and oxidative stress in other rodent models.[179, 180] LHM fed NASH-diet had higher body weight and liver-to-body weight compared to LHM fed a maintenance diet.[181] Moreover, liver histology revealed hepatic steatosis at week 8, and hepatocyte ballooning and fibrosis at week 12.[181] The quantification of plasma lipids is shown in Figure 4.3. Both after eight and twelve weeks, LHM fed NASH-

diet exhibited a dramatic increase in plasma TC, remnant cholesterol, LDL-cholesterol and TGs compared with LHM on maintenance diet and compared with LMM regardless of the diet.

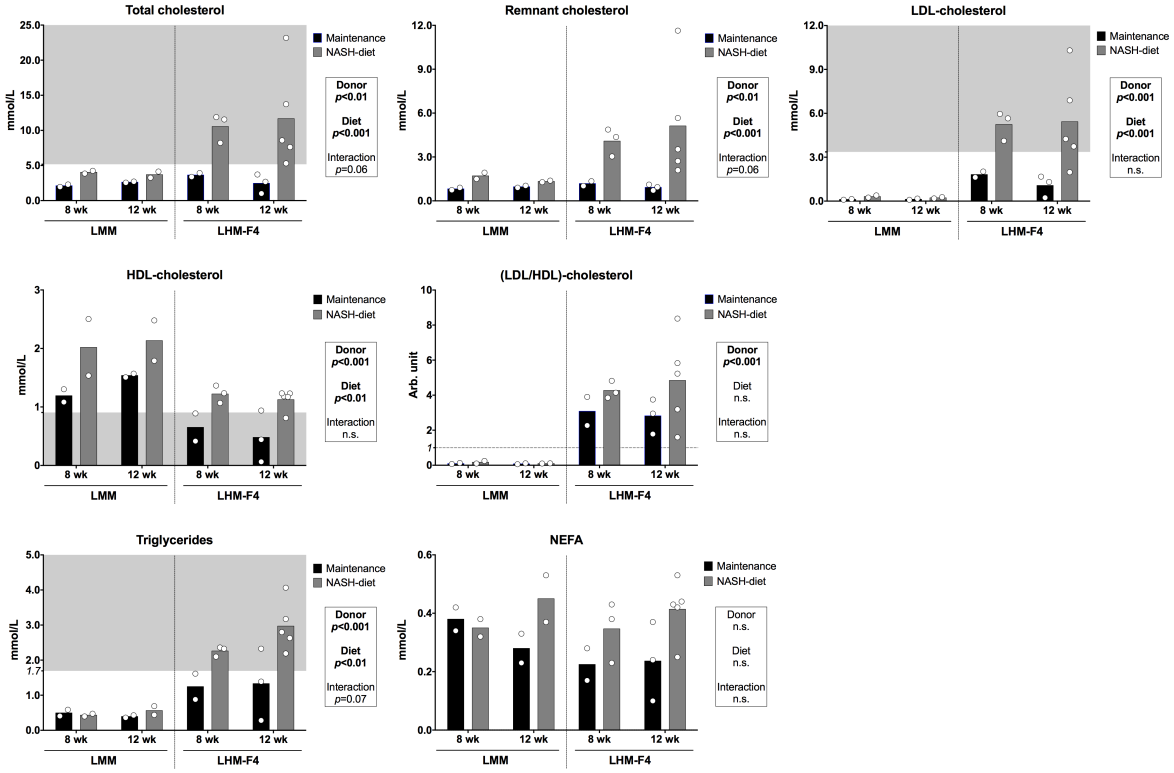

Figure 4.3. Plasma lipids dramatically increase in LHM fed a NASH-diet. *LMM and LHM-F4 were fed maintenance or NASH-diet for 8 or 12 weeks. Lipids were quantified in plasma by routine clinical assays. Bars represent the means of scattered dots. When present, grey areas indicate the values outside the human physiological range. Data were log-transformed before factorial ANOVA using liver humanization (donor), dietary regimen (diet) and dietary feeding time as factors. Because dietary feeding time did not significantly differ in any comparison, the p-values for donor, diet and their interaction are only reported. Abbreviations: NEFA, non-esterified fatty acids.*

To investigate whether the distorted lipid phenotype also resulted in the development of atherosclerosis, we analyzed the cholesterol content in the aorta (Figure 4.4). Despite similar levels of TC and FC in LHM and LMM regardless of the dietary regimen, LHM fed NASH-diet exhibited higher levels of CEs in the aortas, which reflect the formation of atherosclerotic plaques.[182] The development of atherosclerosis in LHM after NASH-diet was confirmed by histological analysis of the aortic valve/bulb, and was more evident in the 12-week group. As shown in Figure 4.5, aortic walls were thicker due to lipid accumulation (panels A and B), and were also enriched of cells positive for CD68 (panel C), indicating macrophage/foam cell infiltration and inflammation.

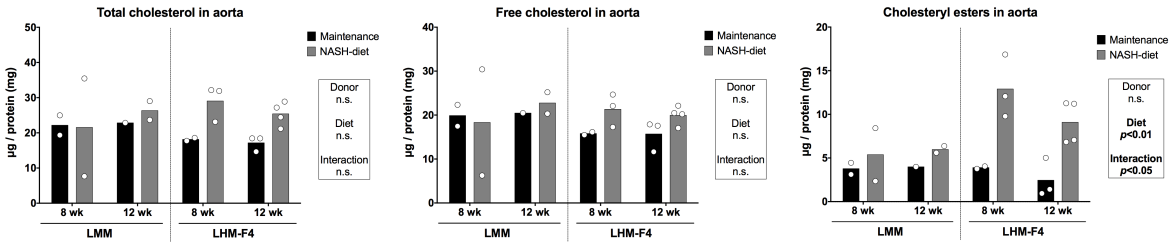

Figure 4.4. LHM fed a NASH-diet accumulate CEs in the descending aorta. *LMM and LHM-F4 were fed maintenance or NASH-diet for 8 or 12 weeks. TC and FC in aortas were quantified by isotope dilution GC–MS after Folch extraction. CEs were calculated as (TC - FC) x 1.67. Bars represent the means of scattered dots. Data were log-transformed before factorial ANOVA using liver humanization (donor), dietary regimen (diet) and dietary feeding time as factors. Because dietary feeding time did not significantly differ in any comparison, the p-values for donor, diet and their interaction are only reported.*

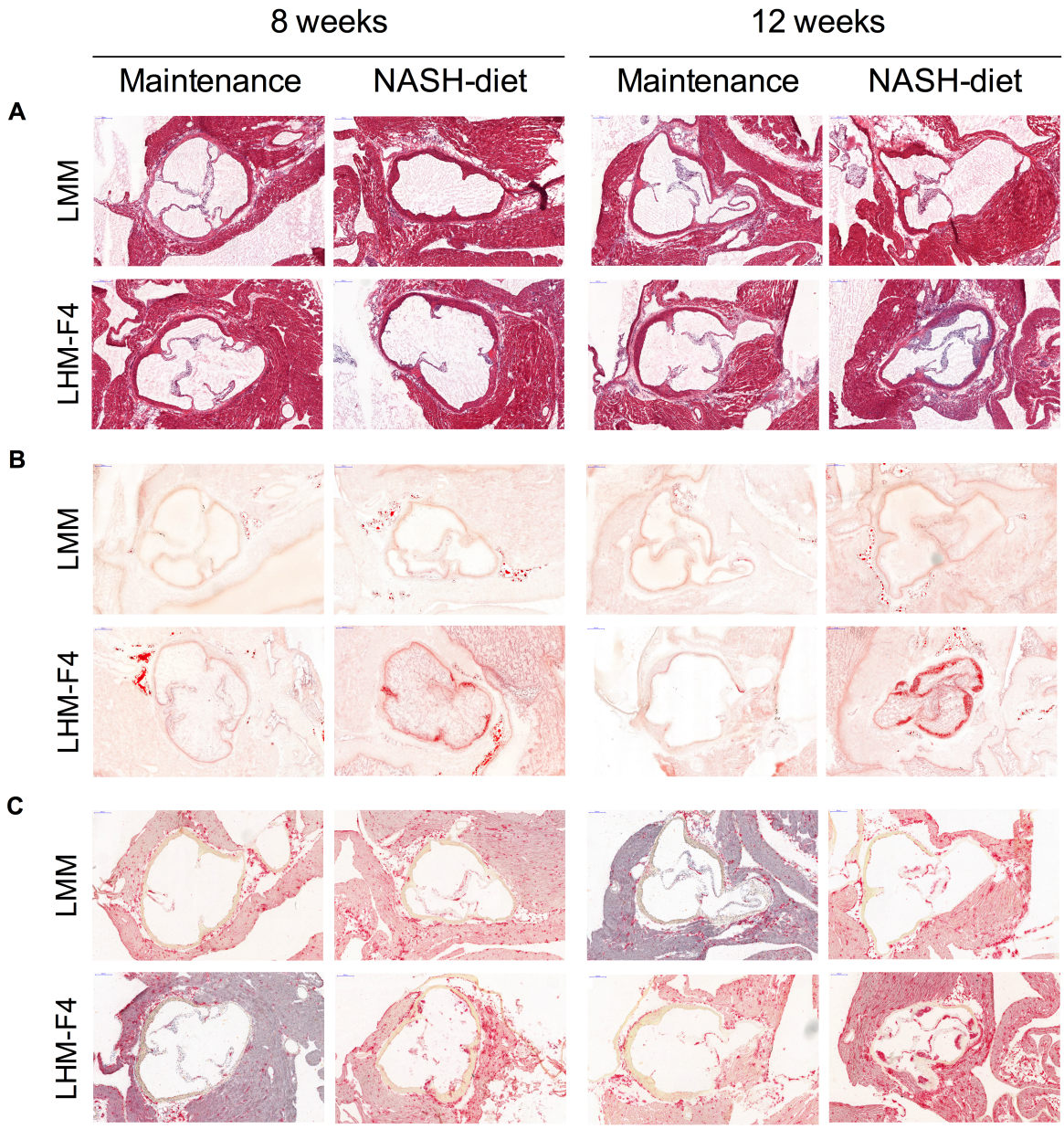

Figure 4.5. LHM develop atherosclerosis on a NASH-diet. *LMM and LHM-F4 were fed maintenance or NASH-diet for 8 or 12 weeks. Hearts were embedded in OCT compound and slices were stained. Representative images for the aortic valve/bulb from one mouse per group are shown (magnification 10X) (**A**) Morphology after H&E stain. (**B**) Lipid accumulation after ORO stain. (**C**) CD68-positive cells.*

From these experiments, it is possible to deduce interesting conclusions on the atherogenic process in LHM. First, LHM develop atherosclerosis despite the absence of CETP, as the regular mouse models for atherosclerosis. This entails that LDL-cholesterol, and especially CEs, drive the atherogenesis independently of the source, and that the beneficial effects of lowering LDL-cholesterol may depend on the corresponding reduction in APOB-containing lipoproteins.[183] Secondly, the atherosclerotic lesions in LHM seem to progress regardless of their absent adaptive immune system, as KO of *Rag2* and *Il2rg* prevents the development of B, T and NK cells.[101, 102] Lymphocytes are considered important for the lesion progression already in the early stages, by secreting both pro- and anti-atherogenic cytokines, and ultimately exacerbating the inflammation of the arterial wall.[5, 184, 185] In LHM, CD68-positive cells (macrophages, but also smooth muscle cells that can take up lipids, turning into foam cells) seem sufficient to drive the atherosclerosis progression. Taken together, these considerations highlight the importance and the causative role of APOB-containing lipoproteins (in particular LDL particles) and of the deposition of cholesterol within the endothelium (triggering the innate immune response) in the development of atherosclerosis, as already stated.[4, 11, 12]

4.1.5 Bile acid metabolism is human-like yet altered in liver-humanized mice

As described in 1.1.4, several differences in BA metabolism are present between humans and mice. LHM fed a HFHSD exhibited a humanized biliary BA composition (**Paper II** and Figure 4.6), similarly to what was observed in LHM under maintenance diet.[86, 107] Again, the similar results regardless of the dietary regimen corroborate the resistance of LHM to HFHSD. The molar fraction of glycine-conjugated BAs – which is low in mice – was significantly higher in LHM compared with LMM. Moreover, the proportion of tauro-DCA was more than double in LHM, in line with the inability of human hepatocytes to reconvert DCA to CA. Finally, the levels of taurine-conjugated MCA – highly represented in LMM – were dramatically reduced in LHM. It has been shown that deletion of *Cyp2c70* in mice virtually inhibits the synthesis of MCA.[88-90] Intriguingly, *Cyp2c70*$^{-/-}$ mice exhibited lower synthesis of BAs and cholesterol, as well as lower levels of LDLR in the liver resulting in an increase of plasma LDL-cholesterol.[90] Although the levels of VLDL- and LDL-cholesterol doubled compared with wildtype mice,[90] this was not sufficient to determine a full humanization of the cholesterol lipoprotein profile as in LHM, presumably due to the maintained ability of the mouse liver to edit APOB mRNA. Nevertheless, these results suggest that MCA in mice play a major role for several differences in liver and lipoprotein metabolism compared with humans, and that a reduction of MCA in LHM may contribute to the humanization of their metabolic phenotype. Further investigations are needed to verify whether these effects are due to inhibition of the hepatic FXR signaling or to other unidentified mechanisms.[88-90]

Lower rates of cholesterol synthesis and dietary intake in humans compared with mice translate into lower rates of BA synthesis in the liver;[33, 45] hence, LHM were expected to have reduced BA synthesis. This was not the case, as LHM were found to have higher hepatic expression of genes involved in BA synthesis compared with humans,[86] and higher levels of

C4, a biomarker for BA synthesis, compared with LMM (**Paper II**). The explanation for the increased BA synthesis in LHM relies on the unresponsiveness of human hepatocytes to the murine intestinal FGF15, which results in abnormal enterohepatic signaling and hepatic BA metabolism, as previously reported.[86, 105, 107]

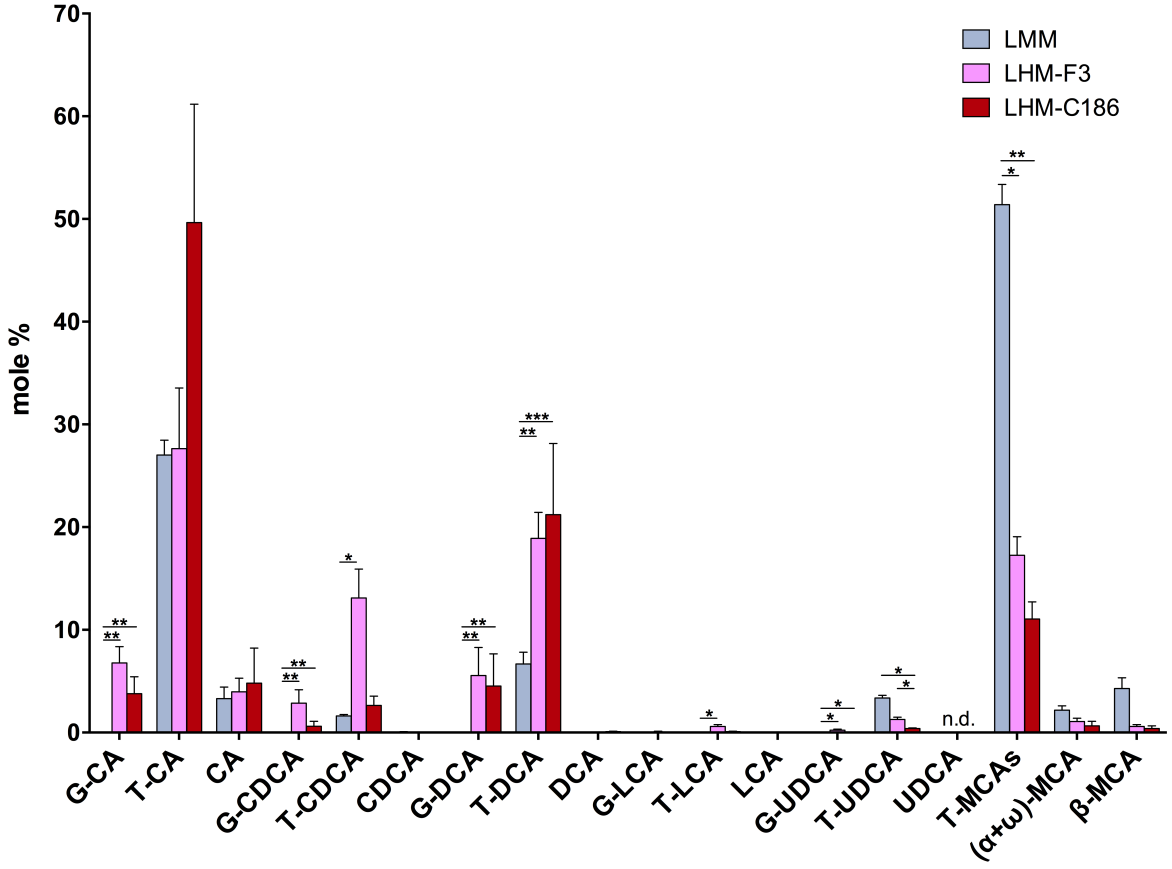

Figure 4.6. Biliary BA composition is humanized in LHM. *BAs were extracted from the emptied gallbladders of LMM, LHM-F3 and LHM-C186, and measured by LC-MS/MS. Mole fractions were calculated, and data are presented as mean and SEM. Data were log-transformed before one-way ANOVA followed by Tukey HSD test. * p<0.05, ** p<0.01 and *** p<0.001. G- and T- indicate the glycine- and taurine-conjugates respectively. Abbreviations: CA, cholic acid; CDCA, chenodeoxycholic acid; DCA, deoxycholic acid; LCA, lithocholic acid; UDCA, ursodeoxycholic acid; MCA, muricholic acid. From* ***Paper II****.*

As discussed in 4.1.3, the levels of TC in the liver did not significantly differ between LHM and LMM on HFHSD, and this was valid also for hepatic CE levels (**Paper II**). However, hepatic levels of CEs only correlated with hepatic TGs and plasma cholesterol levels in LMM (**Paper II**), suggesting a dissociation of CEs from hepatic TG storage and cholesterol secretion occurring in LHM. This is likely resulting from the increased BA synthesis, which led to a higher utilization of cholesterol. The transcriptomic analysis revealed genes important for BA synthesis, uptake, and export to be upregulated in LHM compared with LMM (**Paper II**), including *NPC1L1*, thought to be involved in the uptake of cholesterol from the bile in human – but not in mouse – hepatocytes.[34, 35] The increased BA synthesis in LHM was also accompanied by an increase in lathosterol (**Paper II**), reflecting sustained cholesterol

synthesis. Hence, the correlation between cholesterol and BA biosynthesis observed in LHM suggests that BA synthesis in this model seems to rely more on the newly synthetized cholesterol, the CE pool, and the uptake of cholesterol from the bile, rather than uptake of cholesterol from plasma. In line with this conclusion is the downregulation of the expression of hepatic *SOAT2*, *LDLR*, and *LRP1* observed in LHM compared with LMM (**Paper II**). The positive correlation between cholesterol synthesis and BA synthesis does not normally occurs in humans.[35, 186, 187] This correlation only occurs when the enterohepatic circulation of BAs is interrupted as in patients with bile fistula, in which inhibition of cholesterol synthesis leads to a decrease in BA synthesis.[188] Therefore, LHM seem to resemble the condition of humans having an interrupted enterohepatic circulation of BAs, in line with the unresponsiveness of human hepatocytes to murine FGF15.

The altered BA synthesis resulting from the interrupted BA signaling may also explain – at least in part – the resistance of LHM to HFHSD. While the HFHSD used in this study was low in cholesterol, the NASH-diet with 2% cholesterol prompted LHM to develop severe hepatic steatosis and atherosclerosis. This suggests that high levels of exogenous cholesterol are necessary to overcome the aberrant synthesis of BAs and to induce the development of CMD.

4.1.6 Liver-humanized mice predict human liver pharmacodynamics: the LXR study

After we demonstrated the humanization of lipoprotein and liver lipid metabolism in LHM, we sought to explore the possibility of this model to predict the pharmacodynamic properties of human liver. As a proof of concept, we aimed to test the pharmacological stimulation of the LXR system in LHM (**Paper I**), a strategy considered to have great potential to decrease hyperlipidemia and atherosclerosis.[43] The LXR system has indeed important functions in regulating cholesterol and fatty acid metabolism, and inflammation.[43] Preclinical studies aiming to understand the effects of LXR stimulation showed contrasting results: LXR stimulation was proven to be antiatherogenic in C57BL/6, *Ldlr*$^{-/-}$ and *Apoe*$^{-/-}$ mice,[189, 190] but to increase LDL-cholesterol in hamsters and cynomolgus monkeys, which express the LXR-target gene *CETP*.[191] As expected, the clinical trial testing the effects of the LXR agonist BMS-852927 was prematurely terminated after severe combined hyperlipidemia and TG accumulation in the liver.[192] As shown in **Paper I**, the levels of plasma cholesterol and TG levels (especially in the APOB-containing lipoproteins) dramatically increased in LHM treated with 30 mg/kg/day of the LXR agonist GW3965 for three days. Despite the lack of CETP, LHM recapitulated the hyperlipidemia observed in humans,[192] and could be used to elucidate further (and unexpected) negative effects on liver lipid metabolism after LXR stimulation, as outlined in Figure 4.7.

Conversely to rodents, the LXR-dependent increase of BA synthesis via hepatic *CYP7A1* is absent in humans, hamsters and cynomolgus monkeys.[44, 191] LXR stimulation in LHM actually led to a dramatic decrease of *CYP7A1* mRNA expression and of the classical pathway of BA synthesis (assessed by liver C4 and BA levels), secondary to the upregulation of nuclear receptor subfamily 0 group B member 2 (*NR0B2*, also known as small heterodimer

partner, *SHP*) and *FGF19* (**Paper I**). As a result, cholesterol accumulated in the liver, and decreased the levels of sterol regulatory element-binding transcription factor (*SREBF*) *2* and its target genes, *e.g.*, *HMGCR* and *LDLR*. The downregulation of cholesterol synthesis was confirmed by the reduced levels of hepatic lathosterol and lanosterol (**Paper I**). The excess of hepatic cholesterol was converted in CEs, that accumulated within the liver (**Paper I**). Liver TGs were also increased in LHM after treatment with GW3965, and moderate macrovesicular steatosis was evident from the histological analysis of the liver (**Paper I**). The hepatic TG accumulation in the liver of LHM treated with GW3965 appeared to be mediated by an upregulation of lipid-droplet associated genes (*i.e.*, perilipin 2 and *CIDEC*) and reduced hydrolysis of CEs and TGs. The elevated levels of TGs and CEs in the liver led to increased secretion of cholesterol-rich VLDLs, whereas the increase in plasma TGs (especially in the LDL fraction) seems to derive from a lower *LIPC*/*HL*-mediated hydrolysis.

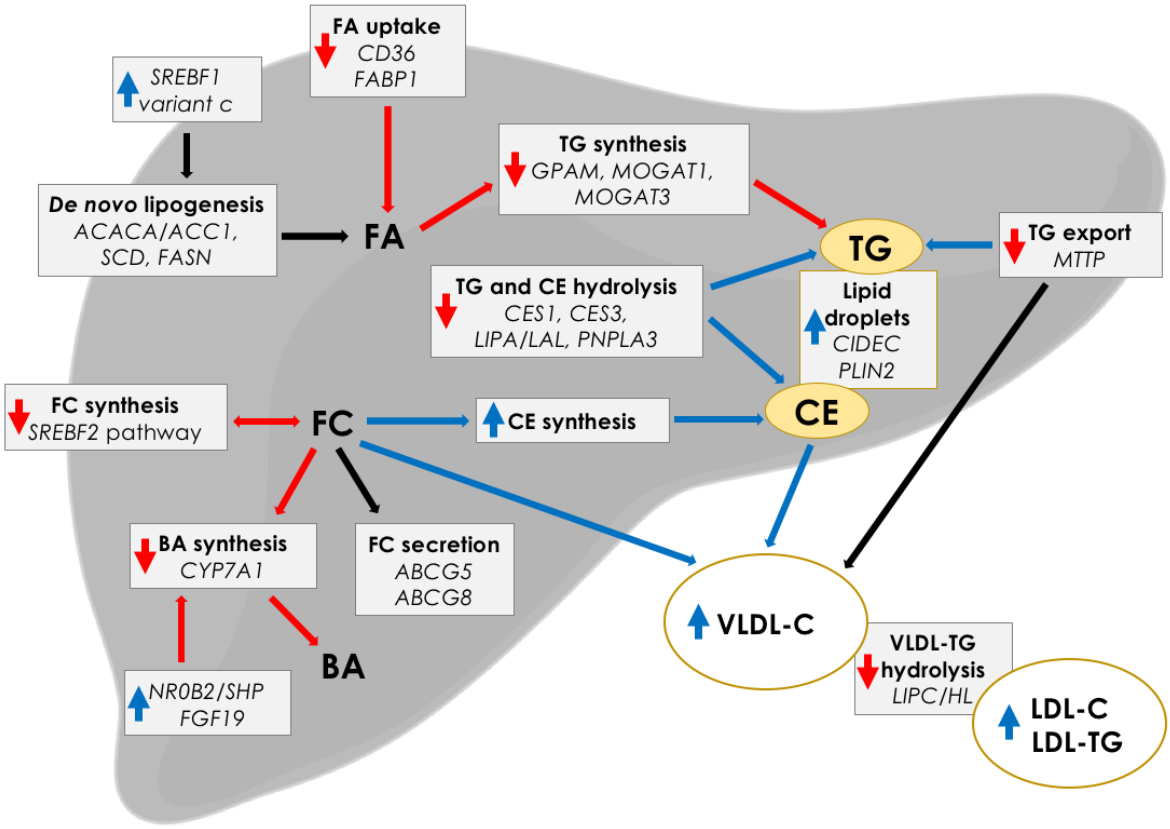

Figure 4.7. The decrease in neutral lipid hydrolysis and BA synthesis explains the increased intracellular and plasma lipids after LXR stimulation in human hepatocytes. *Red, black and blue arrows indicate decrease, no change and increase, respectively. From* ***Paper I****.*

One consideration has to be made on the lipogenic effects of LXR stimulation and on the specificity of the agonist used. The BMS-852927 agonist used in the clinical trial was selective for LXR beta, and was developed to avoid the lipogenic properties of pan-LXR agonists mainly ascribed to hepatic LXR alpha activation from different studies in KO mice.[43, 191, 192] Nevertheless, elevation of plasma and liver TG levels was evident in human

subjects, and similar results were found in LHM treated with the pan-LXR agonist GW3965. These results suggest that LXR beta stimulation is sufficient to exert lipogenic effects in human liver. Moreover, upregulation of hepatic *SREBF1* variant c (a well-known LXR-target gene) in LHM did not affect the target genes involved in *de novo* lipogenesis, indicating the presence of further human-specific mechanisms that were not identified in preclinical studies using conventional mouse models.

Finally, another aspect to consider is the increase of serum CEC by aqueous diffusion and ABCA1 found in LHM after LXR stimulation (**Paper I**), which represents the increased ability of all serum components (*i.e.*, lipoproteins, APOs and albumin) to accept cholesterol from tissues via certain mechanisms. Although the increase of CEC has been considered as one of the main atheroprotective properties of LXR stimulation (especially when referred to HDLs), this effect in humans may be dampened by the unfavorable lipoprotein and liver lipid phenotype.

4.2 HOW TO IMPROVE THE HUMAN RELEVANCE OF LIPOPROTEIN AND INTRACELLULAR METABOLISM IN HEPATOCYTE-LIKE CELL LINES

4.2.1 Culturing with human serum

HepG2 and Huh7.5 are two human hepatocyte-like cell lines derived from human hepatoblastoma and hepatocellular carcinoma, respectively.[172, 173] HepG2 cells have long been used as a preclinical model for studies of hepatic lipid metabolism, whereas Huh7.5 cells have more recently been used for these studies and are thus less well characterized. However, both cell lines exhibit major differences compared with human hepatocytes *in vivo* that limit their relevance for studies on human pathophysiology. For instance, HepG2 cells cultured under standard conditions with 10% FBS secrete aberrant lipoproteins and display HDL-sized APOB-containing lipoproteins in the cell medium.[113] Hence, it is necessary to explore and validate new strategies to improve the usefulness of hepatocyte-like cellular models for studies of hepatic lipid metabolism. Culturing of HepG2 cells in medium supplemented with 2% HS instead of 10% FBS increased the levels of APOB-containing LDL-sized and APOA1-containing HDL-sized particles, tremendously improving the lipid phenotype of HepG2 cells.[113] As we and others previously found Huh7.5 cells to have higher levels of intracellular TGs,[193, 194] and thus have the potential to be better suited as model for hepatic steatosis/NAFLD, as well as to better characterize this cell line, in **Paper III** we also assessed the effects of culturing Huh7.5 with FBS and HS. Supplementation of the culturing medium with HS did not improve the lipoprotein phenotype of Huh7.5, as lower levels of cholesterol in the medium were found compared with cells cultured with FBS (**Paper III**). Similarly, the levels of TGs secreted in the medium (especially in the LDL fraction) increased in HepG2 cells cultured in HS,[113] but were lower in Huh7.5 cells compared with cells cultured with FBS (**Paper III**). In line with these results, HepG2 cells cultured in medium supplemented with HS had higher levels of APOB in the medium,[113] but no changes were found in Huh7.5 cells compared with cells cultured with FBS (**Paper III**). Culturing with HS resulted in decreased levels of intracellular FC, CEs and TGs in both cell lines (**Paper III**).

This is particularly important in HepG2 cells, as an impaired intracellular lipid mobilization appears to be responsible for the secretion of smaller APOB-containing lipoproteins.[113] However, the most evident effect on the levels of intracellular TGs was observed in Huh7.5 cells, in which intracellular TG levels decreased by about 75% and thereby reached similar levels as observed in HepG2 cells, although this was not associated with an improved lipoprotein profile. Finally, supplementation of the culturing medium with HS restored the levels of BAs in the medium in HepG2 cells,[113] whereas BA levels were too low to be accurately detected in Huh7.5, regardless of the supplementation of the culturing media (**Paper III**). The improvements observed in HepG2 cells cultured with HS are likely dependent on the increased expression of important genes of lipid metabolism and hepatocyte differentiation.[113] These results highlight the importance of both the culturing conditions and the cell line utilized as *in vitro* model for human pathophysiology. Collectively, these findings suggest HepG2 cells cultured in HS to better reflect the physiological condition of human hepatocytes *in vivo*. Conversely, culturing of Huh7.5 cells under standard conditions, or potentially in media supplemented with exogenous lipids,[118] may be better suited for studies of hepatic steatosis/NAFLD.

4.2.2 Genome editing

4.2.2.1 Knockout of sterol O-acyltransferase 1

Because impaired intracellular lipid mobilization is associated with lower secretion of APOB-containing lipoproteins, we sought to improve this aspect in HepG2 and Huh7.5 cells. As described in 1.3.1.2, two isoforms of *SOAT* catalyze the formation of intracellular CEs from fatty acids and cholesterol: *SOAT1*, ubiquitously expressed, and *SOAT2*, which is solely expressed in hepatocytes and enterocytes, and determines the amount of CEs secreted in APOB-containing lipoproteins.[123, 124] Conversely to normal hepatocytes *in vivo*, which only express *SOAT2*, both isoforms are expressed in HepG2, Huh7.5 and also in PHH. Because the presence of *SOAT1* in hepatocyte-like cell lines may contribute to their distorted lipoprotein and lipid phenotype, in **Paper III** we used the CRISPR technology to KO *SOAT1* in HepG2 and Huh7.5 cells, generating models better resembling the situation of human hepatocytes *in vivo* expressing only *SOAT2*. Moreover, we cultured both unedited and *SOAT2*-only cells with either FBS or HS to assess whether the combination of *SOAT1*-KO with culturing with HS could further improve the phenotype of HepG2 and Huh7.5 cells. *SOAT2*-only-HepG2 cells exhibited even higher levels of cholesterol and TGs in all lipoprotein fractions in the medium when cultured with HS (**Paper III**). Conversely, the levels of cholesterol and TGs in the medium in *SOAT2*-only-Huh7.5 cells were lower compared with the unedited cells, independently of the culturing condition (**Paper III**). Similarly, the levels of APOB in the cell medium further increased when culturing *SOAT2*-only-HepG2 cells with HS, whereas the opposite was observed in *SOAT2*-only-Huh7.5 cells (**Paper III**). Although *SOAT1*-KO had no effect on intracellular CE and TG levels in HepG2 cells cultured with FBS, it could restore the decreased levels observed in HepG2 cells cultured with HS (**Paper III**). Conversely, *SOAT1*-KO in Huh7.5 cells only decreased the

intracellular CE levels, and the culturing with HS had the strongest effect in decreasing both CEs and TGs independently on the genotype (**Paper III**). Finally, the response to pharmacological cholesterol synthesis inhibition in HepG2 cells was higher when these cells were cultured with HS rather than with FBS.[113] This was further improved in *SOAT2*-only-HepG2 cells cultured with HS, which exhibited a full transcriptional response to the treatment with atorvastatin (**Paper III**). Again, the further improvements observed in *SOAT2*-only-HepG2 cells cultured with HS are likely dependent of an additional increase in the expression of important genes involved in lipid metabolism and hepatocyte differentiation (**Paper III**). Taken together, these results underline that: (a) *SOAT1* expression in hepatocyte-like cell lines contributes to their distorted lipid and lipoprotein phenotype, (b) the different effects seen with HS and *SOAT1*-KO (alone or in combination) strictly depend on the cellular model and the origin/genetic background, and (c) *SOAT2*-only-HepG2 cells cultured with HS represent a further improved *in vitro* model for studies of human hepatic lipid metabolism.

4.2.2.2 Knockout of cell death-inducing DFFA-like effector c

Genetic depletion of *Soat2* in mice ameliorates dietary-induced hepatic steatosis.[125, 126] Interestingly, we noticed this to be associated with a dramatic impairment of the diet-induced upregulation of hepatic *Fsp27* expression (unpublished data). This corroborates the hypothesis that the diet-induced increase in *Fsp27*/*CIDEC* plays a major role for the development of hepatic steatosis. After successfully using the CRISPR technology in human hepatocyte-like cell lines to KO *SOAT1*, in **Paper IV** we used the same methodology to generate a *CIDEC*-KO-HepG2 cellular model in order to investigate how *CIDEC* affects hepatic lipid and carbohydrate metabolism in human hepatocytes. The preliminary functional validation of *CIDEC*-KO-HepG2 cells revealed similar levels of intracellular cholesterol and TGs compared with the unedited HepG2 cells (**Paper IV**). The scarce effect on intracellular lipids are probably due to the low basal levels of intracellular TGs in HepG2 cells.[113] However, the analysis of gene expression indicated that *CIDEC*-KO may affect fatty acid metabolism and TG storage/secretion in HepG2 cells, although further investigations are needed to characterize the effects of *CIDEC*-KO on lipid and carbohydrate metabolism in HepG2 cells under different conditions.

5 CONCLUSION AND FUTURE PERSPECTIVE

The primary objective of this thesis was to develop and characterize new advanced *in vivo* and *in vitro* preclinical models of greater relevance for human hepatic lipid and lipoprotein metabolism.

As shown in **Papers I** and **II**, LHM repopulated with human hepatocytes from different donors exhibited a humanized lipoprotein and BA metabolism. LHM not only appeared to be an improved human translatable model, but also allowed to reshape the physiological paradigm of why humans are LDL animals and mice are non-LDL animals. We were also able to characterize at molecular level the pharmacodynamic effects of LXR stimulation on human hepatocytes, and thereby in depth understand the negative lipid outcomes of a previous clinical trial testing an LXR agonist. However, despite the improved liver metabolism, several limitations are still present in this animal model. For instance, not all human liver-specific factors were recapitulated, for example these mice lack expression of CETP and exhibit aberrant BA metabolism. While these caveats may be theoretically improved by humanizing the immune system as well as inserting human genes,[104, 105] other caveats like the variable liver chimerism, the mouse peripheral metabolism and the human donor/mouse recipient cell interaction will always affect the complete humanization of LHM metabolism. Hence, in order to fully develop the potential of LHM for human pathophysiological studies, further improvements are required to definitively abandon the poorly relevant classical mouse models.
An unequivocal hallmark of LHM is that the lipoprotein and liver metabolic phenotype is affected by the hepatocytes of the human donors. Several examples were indeed found throughout their characterization. Therefore, the combination of this aspect with the improved human translatability allows LHM to be used in several personalized medicine settings.
We also explored the possibility of LHM to be used as a model of diet-induced CMD. LHM appeared to be resistant to HFHSD. However, LHM developed severe hepatic steatosis and atherosclerosis after eight-week feeding with NASH-diet despite the immune deficiency. It will be very important to assess whether these differences depend only on the diet composition or on other factors. As a matter of fact, we and our collaborators are now investigating whether the resistance of LHM to HFHSD is associated with glucose metabolism/insulin sensitivity, oxidative metabolism and in the crosstalk between the humanized liver and the murine host.

In **Papers III** and **IV** we assessed whether the culturing with HS and/or the CRISPR-genome editing technology could improve the relevance of human hepatocyte-like cells lines for studies of human liver metabolism. Culturing with HS dramatically improved the lipid and lipoprotein phenotype of HepG2 cells. Conversely, Huh7.5 cells seem to be better suited for studies of lipid accumulation due to their higher basal levels of intracellular TGs. Despite the improved phenotype of these cells following culture with HS, both *SOAT1* and *SOAT2* were expressed in these cell lines, whereas human hepatocytes only express *SOAT2 in vivo*. Hence, none of these *in vitro* models cultured under standard conditions resembles the human

situation *in vivo*. The newly generated *SOAT2*-only-HepG2 cell line cultured with HS had a dramatic improved lipid phenotype. These cells may therefore be used as a new preclinical standard *in vitro* for studies of the metabolism of human hepatocytes *in vivo*. To extend the characterization of this model, the comparison between *SOAT2*-only-HepG2 and PHH is ongoing.

We have also generated a *CIDEC*-KO-HepG2 cell line to study the effects of *CIDEC* inhibition on hepatic lipid and carbohydrate metabolism. The preliminary characterization of these cells at lipid level showed altered expression of genes involved in fatty acid metabolism and TG storage/secretion, but these alterations were not accompanied by significantly changed levels of intracellular TGs. Hence, we are planning to culture *CIDEC*-KO-HepG2 cells with HS and/or under lipid-laden conditions to validate this cellular model and to assess the potential pathological roles of *CIDEC* in hepatic lipid and carbohydrate metabolism. Because Huh7.5 cells may be a better model for studies of hepatic steatosis/NAFLD, we have also knocked out *CIDEC* in Huh7.5 cells and we are currently screening the edited clones. As for *CIDEC*-KO-HepG2 cells, the successfully edited *CIDEC*-KO-Huh7.5 cells will be thoroughly characterized. As a final note, the versatility of the CRISPR technology allows several genes to be edited in the same cell line: hence, KO of *CIDEC* in *SOAT2*-only-HepG2 and/or *SOAT2*-only-Huh7.5 cells may provide an even better *in vitro* model to investigate the effects of CIDEC on human hepatic lipid metabolism and hepatic steatosis.

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