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ROLE OF CHOLESTEROL METABOLISM IN HEPATIC STEATOSIS AND GLUCOSE TOLERANCE

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Role of cholesterol metabolism in hepatic steatosis and glucose tolerance
THESIS FOR DOCTORAL DEGREE (Ph.D.)

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بسم الله الرحمن الرحيم

To the dedicated man who has struggled and persevered to lighten the road that I now walk as my own.

My Father Salih

ABSTRACT

The liver is the central organ for lipid, lipoprotein and glucose homeostasis, thus hepatic metabolic disturbances can predispose individuals to develop cardiometabolic disorders (CMD) such as atherosclerotic cardiovascular diseases (ASCVD), type 2 diabetes mellitus (T2DM), and nonalcoholic fatty liver disease (NAFLD).The overall aim of this thesis was to expand the knowledge on how genetic and pharmacological interventions on hepatic and intestinal cholesterol metabolism could affect the pathophysiology of CMD.

Papers I and **II**: Acyl-Coenzyme A:cholesterol acyltransferase 2 (ACAT2, encoded by the *Soat2* gene) is exclusively expressed in hepatocytes and enterocytes and catalyzes the biosynthesis of cholesteryl esters from cholesterol and long-chain fatty acids. Previous studies in mice model have shown that loss of ACAT2 activity protects from atherosclerosis, diet-induced hypercholesterolemia, and dietary cholesterol-driven hepatic steatosis. Here, we aimed to dissect the potential molecular mechanisms by which genetic depletion of *Soat2* could affect the pathophysiology of hepatic steatosis and insulin sensitivity, independently of dietary regimens. We found that depletion of *Soat2* significantly reduces hepatic steatosis and improves glucose tolerance, independently of high levels of cholesterol in the diet. We proposed the downregulation of hepatic *de novo* lipogenesis; DNL (lipid synthesis from glucose), GLUT2 membrane protein and *Cd36* mRNA levels, as main mechanisms by which *Soat2* depletion improves CMD. Dampening induction of CIDEC/FSP27 mRNA and protein levels in the severe fatty liver is another potential mechanism. Thus, cholesterol esterification by ACAT2 seems to be linked to hepatic steatosis and glucose homeostasis. Taken together, our study strongly supports ACAT2 inhibition as a promising target to treat CMD.

Papers III and **IV**: Ezetimibe and simvastatin inhibit cholesterol absorption and cholesterol synthesis, respectively. Adding ezetimibe to simvastatin therapy has been shown to result in an additional absolute risk reduction of death from ASCVD, particularly among patients with T2DM (IMPROVE-IT trial). In **Paper III**, we aimed to investigate the potential positive effects of cholesterol absorption and/or cholesterol synthesis inhibition on remnant particles, the binding to arterial proteoglycans (PG), and biliary lipid compositions as well as hepatic sterol regulatory element-binding protein 2 (SREBP2) target genes. Combined therapy resulted in athero-protective changes on remnant and apoB-lipoprotein particles, and on the affinity for arterial PG. In **Paper IV,** we aimed to further characterize the effects by the addition of ezetimibe to simvastatin therapy on the hepatic transcriptional signature to uncover potential beneficial responses on different metabolic pathways in humans. We identified a total of 260 reliable genes to be altered during the different treatments. Gene ontology and pathways analysis displayed involvement of the combined therapy in classical antibody-mediated complement activation. In view of individual genes, adding ezetimibe to simvastatin seems to affect the predisposition to hepatic steatosis and NAFLD, and improve the glucose tolerance; however functional validation in bigger cohorts is needed.

Collectively, our data might explain the decrease of ASCVD events reported in the IMPROVE-IT and SHARP trials, especially in T2DM patients. Hence, we propose the addition of ezetimibe to simvastatin therapy as an optimal intervention for lipid disorders characterized by elevated remnant-cholesterol (such as T2DM) to improve the outcome of CMD.

Key words: ACAT2, ASCVD, CIDEC/FSP27, CMD, Ezetimibe, GLUT2, NAFLD, PG binding, remnant-cholesterol, T2DM, Simvastatin, and SREBP2.

LIST OF SCIENTIFIC PAPERS

This doctoral thesis is based on the following original papers

I. **Ahmed O**, Pramfalk C, Pedrelli M, Olin M, Steffensen KR, Eriksson M, Parini P. Genetic depletion of *Soat2* diminishes hepatic steatosis via genes regulating *de novo* lipogenesis and by GLUT2 protein in female mice. *Dig Liver Dis*, 2018.

II. Pramfalk C, **Ahmed O**, Härdfeldt J, Pedrelli M, Vedin LL, Steffensen KR, Eriksson M, Parini P. Genetic depletion of the *Soat2* gene improves glucose tolerance by reducing hepatic steatosis in male mice. *Manuscript*

III. **Ahmed O***, Littmann K*, Gustafsson U, Pramfalk C, Öörni K, Larsson L, Minniti M E, Sahlin S, Camejo G, Parini P*, Eriksson M*. Ezetimibe in combination with simvastatin reduces remnant-cholesterol without affecting biliary lipid concentrations in gallstone patients. *Journal of the American Heart Association*. 2018; 7:e009876
*These authors contributed equally to this work.

IV. **Ahmed O**, Mukarram AK, Pirazzini C, Marasco E, Minniti M E, Gustafsson U, Sahlin S, Pramfalk C, Garagnani P, Daub CO, Eriksson M, Parini P. Hepatic transcriptional response to combination of ezetimibe with simvastatin in gallstone patients. *Manuscript*

CONTENTS

LIST OF ABBREVIATIONS

ABC	ATP-binding cassette
ABCA1	ATP-binding cassette transporter A1
ABCB4	ATP-binding cassette transporter B4
ABCB11	ATP-binding cassette transporter B11
ABCG5	ATP-binding cassette transporter G5
ABCG8	ATP-binding cassette transporter G8
ACAT	Acyl-coenzyme A:cholesterol acyltransferase
ACC	Acetyl-CoA carboxylase
ASO	Antisense oligonucleotide
AMP	Adenosine monophosphate
AMPK	AMP-activated protein kinase
Apo	Apolipoprotein
ASCVD	Atherosclerotic cardiovascular diseases
ATP	Adenosine triphosphate
BA	Bile acid
CD36	Cluster of differentiation 36
cDNA	Complementary DNA
CE	Cholesteryl ester
CETP	Cholesteryl ester transfer protein
ChREBP	Carbohydrate-responsive regulatory element-binding protein
CIDE	Cell death-inducing DFFA-like effector
CMD	Cardiometabolic disorder
CoA	Coenzyme A
CPT1	Carnitine palmitoyl transferase 1
CVD	Cardiovascular disease
CYP7A1	Cholesterol 7α-hydroxylase
DAG	Diacylglycerol
DNL	De novo lipogenesis
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic Reticulum

FC	Free cholesterol
FSP27	Fat-specific protein 27
GLUT2	Glucose transporter 2
GO	Gene ontology
GTT	Glucose tolerance test
GWAS	Genome-wide association study
HCC	Hepatocellular carcinoma
HCD	High-carbohydrate diet
HDL	High-density lipoprotein
HFD	High-fat diet
HL	Hepatic lipase
HMG-CoA	3-Hydroxy-3-methylglutaryl-Coenzyme A
HMGCR	3-Hydroxy-3-methylglutaryl-Coenzyme A reductase
HMGCS	3-Hydroxy-3-methylglutaryl-Coenzyme A synthase
HNF	Hepatocyte nuclear factor
HOMA-IR	Homeostatic model assessment of insulin resistance
IDL	Intermediate-density lipoprotein
IMPROVE-IT	Improved reduction of outcomes: vytorin efficacy international trial
INSIG	Insulin induced gene
ITT	Insulin tolerance test
IR	Insulin resistance
IRS2	Insulin receptor substrate
LCAT	Lecithin-cholesterol acyltransferase
LD	Lipid droplet
LDL	Low-density lipoprotein
LDLR	Low-density lipoprotein receptor
LFC	Logarithmic-fold change
LPL	Lipoprotein lipase
LRP1	Low-density lipoprotein receptor-related protein 1
LXR	Liver X receptor

mRNA	Messenger RNA
MS	Mass spectrometry
MTP	Triglyceride transfer protein
NAFLD	Nonalcoholic fatty liver disease
NASH	Nonalcoholic steatohepatitis
NEFA	Non-esterified fatty acid
NPC1L1	Niemann-Pick C1-Like protein 1
PCA	Principle component analysis
PG	Proteoglycans
PKC	Protein kinase C
PL	Phospholipid
Plin2	Perilipin 2
PLTP	Phospholipid transfer protein
PUFA	Polyunsaturated fatty acid
PPAR	Peroxisome proliferator-activated receptor
RCT	Reverse cholesterol transport
SCAP	Sterol regulatory element binding protein cleavage activating protein
sdLDL	Small dense LDL
SEC	Size-exclusion chromatography
SHARP	Study of heart and renal protection
SOAT2	Sterol O-acyltransferase 2
SR-BI	Scavenger Receptor class B type I
SREBP	Sterol regulatory element binding protein
T2DM	Type 2 diabetes mellitus
TG	Triglyceride
TICE	Transintestinal cholesterol excretion
VLDL	Very low-density lipoprotein
VLDLR	Very low-density lipoprotein receptor
WT	Wild type

1. BACKGROUND

The liver is the key organ for regulation of lipid, lipoprotein and glucose metabolism, thus hepatic metabolic derangements can predispose individuals to develop cardiometabolic disorders (CMD). CMD are a cluster of metabolic disturbances leading to development of atherosclerotic cardiovascular diseases (ASCVD), type 2 diabetes mellitus (T2DM) and nonalcoholic fatty liver disease (NAFLD).

1.1 NONALCOHOLIC FATTY LIVER DISEASE (NAFLD)

NAFLD has become a global health problem and the prevalence is estimated at 25% of the adult population and its clinical and economic burdens is already great and expected to rise further[1]. No evidence-based treatment for NAFLD is approved yet. Hence, the development of new effective therapeutic strategies is needed. NAFLD is defined as intracellular triglyceride (TG) content of >5% of liver weight or volume[2, 3] in individuals consuming less than 20 g alcohol per day after exclusion of concomitant liver disease etiologies[1, 4]. The exclusion of alcohol use is important in the diagnosis of NAFLD since the most common cause of TG accumulation leading to secondary steatosis is significant alcohol intake (>21 drinks/week in men and >14 drinks/week in women according to the U.S. guideline[5]).

NAFLD is a term used to describe the histological spectrum of conditions spanning from simple hepatic steatosis to liver damage. The chronic combination of lipid accumulation with low-grade inflammation makes hepatic steatosis to progress to NASH[4], which is characterized by hepatocyte ballooning and fibrosis[1]. NASH can progress to cirrhosis, hepatocellular carcinoma (HCC) and liver failure and is an increasing indication for liver transplantation[1, 6]. Some, but not all, of the subjects having NAFLD will progress to the more severe liver conditions[1]. In addition to liver-related mortality and morbidity, NAFLD is associated with and likely play a causal role in the development of extrahepatic manifestations such as ASCVD and chronic kidney disease[1, 4]. Thus NAFLD is a frequent co-morbidity in CMD.

1.1.1 Hepatic steatosis

As mentioned above, the hallmark of NAFLD is hepatic steatosis that is defined as the non-physiological accumulation of TG inside hepatic lipid droplets (LDs). TG accumulation in the liver determines adverse metabolic consequences affecting glucose, fatty acid (FA), and lipoprotein metabolisms, as well as promoting an inflammatory state[2].

Although the molecular mechanisms causing hepatic steatosis and its progression to the more severe stages are yet not defined, hepatic steatosis develops as a consequence of an imbalance between FA input (the rate of synthesis, *de novo* lipogenesis, and uptake from the circulation) and FA output (the rate of oxidation and export)[2]. Therefore, understanding the underlying metabolic alterations that result in excess hepatic TG accumulation may reveal therapeutic strategies for the treatment of hepatic steatosis and the accompanying metabolic disturbances.

1.1.1.1 *De novo lipogenesis (DNL)*

The liver uses non-lipid precursors such as glucose as substrate to synthesize FAs through a cytosolic enzymatic system. Acetyl-CoA carboxylase (ACC) catalyzes the rate-limiting step, the conversion of acetyl-CoA to malonyl-CoA. Mice with constitutively active ACC have higher hepatic DNL and develop hepatic steatosis, glucose intolerance and insulin resistance (IR)[7]. Conversely, knockdown or liver-specific knockout of ACC isoforms protects mice from the development of hepatic steatosis[8, 9].

Hepatic DNL is mainly regulated at transcriptional level. Insulin and glucose respectively activate the sterol regulatory element-binding protein 1c (SREBP1c)[10] and carbohydrate-responsive regulatory element-binding protein (ChREBP)[11] transcriptional factors, which in turn and independently from each other activate the transcription of almost all the genes involved in DNL.

Hepatic DNL represents less than 5% of hepatic TG accumulation in healthy subjects[12]. However, this situation changes in steatotic livers in which DNL has been estimated to be responsible for 15-25% of the TG accumulation[12, 13]. Hence, an enhanced DNL in the liver is considered one of the major metabolic derangements in patients with NAFLD[12, 14]. Also, pharmacological inhibition of key enzymes involved in DNL reverses NAFLD in rodents and humans[15, 16]. Furthermore, it is suggested that the enhanced DNL might predispose to the development of HCC, as DNL inhibition suppresses HCC in rats[17].

The widely prescribed T2DM drug metformin mediates its insulin-sensitizing effects through regulation of DNL and FA oxidation[7]. Fullerton and his colleagues gave evidence that chronic metformin treatment reduces hepatic DNL and steatosis by activating AMP-activated protein kinase (AMPK) and consequently inhibiting both ACC1 and ACC2[7]. This makes metformin a good candidate for treating IR and steatosis in NAFLD patients, however positive effects have been shown in some but not all clinical trials[18].

1.1.1.1.1 <u>Glucose transporters (GLUTs)</u>

The GLUT family members are a group of highly related membrane proteins[19]. GLUT2 transports glucose across the hepatic plasma membrane in a bidirectional manner[20] and contributes to homeostasis of intra-hepatic glucose, the main substrate of DNL. Hepatic GLUT2 is also as DNL regulated by SREBP-1c[21] which might indicate their coordinate function. Hence, increased hepatic GLUT2 protein levels result in hepatic steatosis associated with increased DNL[22] and lowering of hepatic GLUT2 improved hepatic steatosis[23]. It has been shown that peroxisome proliferator-activated receptor (PPAR) alpha agonists improve hepatic steatosis and IR in diet-induced obese mice not only by increasing FA oxidation but also through a reduction of SREBP1c and consequently hepatic GLUT2 and DNL[24]. This data suggest the enhanced hepatic gluconeogenesis and increased GLUT2 protein as main contributors in hyperglycemia and IR in this model.

1.1.1.2 *FA uptake*

The rate of FA uptake in the liver depends on the circulating plasma levels of non-esterified FA (NEFA) released from extra-hepatic tissues, particularly from the adipose tissue. Donnelly KL and his colleagues[13] have demonstrated that the NEFA in circulation are the major contributor to hepatic TG (~ 60%) in fasted NAFLD patients. Once IR arises, a vicious circle between the liver and the adipose tissue is formed. IR causes a dysfunctional adipose tissue in which the rates of lipolysis are increased[25] due to the compromised insulin signaling. As a consequence, the adipose tissue increase release of NEFA is involved in the pathogenesis of hepatic steatosis and NAFLD[26]. Hence, treating patients with NAFLD with insulin-sensitizing agents have shown promising results[27-29].

1.1.1.2.1 Cluster of differentiation 36 (CD36)

CD36 is a glycoprotein expressed on the membrane of different cell types including hepatocytes, adipocytes and myocytes. CD36 can bind to and take up long-chain FA, oxidized lipids, and lipoproteins[30]. Gene and protein levels of CD36 are decreased in adipose tissues but increased in the skeletal muscles in individuals with hepatic steatosis[31], indicating that tissue distribution of CD36 may redirect the uptake of circulating NEFA from adipose to other tissues.

Hepatic expression of CD36 has been reported to be correlated with hepatic steatosis, hyperinsulinemia and IR in animal model[32]. NAFLD patients had increased serum NEFA levels[33] with the preferential distribution of fat into the liver due to increased expression of hepatic CD36[34]. Both changes in adipose tissue lipolytic activity and increased expression of hepatic CD36 seem to contribute in the development of hepatic steatosis and NAFLD.

1.1.1.3 *FA oxidation*

The liver performs several complex metabolic processes for which ~ 20% of the total resting energy expenditure is consumed[2]. FA and amino acid oxidation are estimated to provide about 90% of the basal hepatic energy consumption, although FA oxidation is reduced during the fed state[2]. FA oxidation produces acetyl-CoA which can either proceed to oxidation for hepatic energy provision or be converted to ketone bodies which provide energy to other tissues[35].

FA oxidation is transcriptionally regulated by PPAR alpha and takes place primarily in the mitochondria, and to a lesser extent in peroxisomes. PPAR alpha agonists have been shown to improve hepatic steatosis and NAFLD in animal models by increasing FA oxidation[24, 36].

Experimental knockout of mitochondrial oxidative enzymes induced hepatic steatosis[37] whereas increasing their expression or activity improves steatosis[36, 38]. Moreover, administration of recombinant adiponectin in ob/ob mice alleviates hepatic steatosis by increasing carnitine palmitoyl transferase 1 (CPT1) activity and hepatic FA oxidation, while

reducing ACC activity and DNL[38]. However, studies of FA oxidation in patients with NAFLD present conflicting results (for review see[39]).

1.1.1.3.1 DNL activity regulates FA oxidation

The transport of FA from the cytosol into the mitochondrial matrix is regulated by CPT1[40]. As mentioned above, ACC catalyzes the first and committed step of DNL which produces malonyl-CoA, an allosteric inhibitor of (CPT1)[35]. Therefore, ACC regulates both DNL and FA oxidation and maintains their inverse relationship. The body metabolic status regulates the hepatic fuel selection through modulation of key enzymes by allosteric interactions. For example, cytoplasmic levels of citrate sense the fed state and activate ACC, while elevated palmitoyl-CoA levels, an indicator of the fasting state, inhibit ACC[41]. Hence, ACC activity plays a central role in the metabolic fate of intracellular FA as well as in the shift between carbohydrate and FA consumption as energy sources[35].

1.1.1.3.2 Cell death-inducing DFFA-like effector c (CIDEC)

Recent studies have explored the crucial role of LD proteins on lipid metabolism, transport, and signaling. *CIDEC* and its mouse orthologous fat-specific protein 27 (*Fsp27*) encodes for a LD protein that is highly expressed in brown and white adipose tissues. CIDEC/ FSP27 is enriched at the contact points between LDs, promoting their fusion into bigger ones[42]. FSP27 is expressed at low levels in the liver; however, its β isoform is highly expressed[43] during fasting[44] and diet-induced hepatic steatosis[45] and are regulated by PPAR alpha and PPAR gamma, respectively. Hence, FSP27 seems to play a role in hepatic metabolic adaptations to both physiological (fasting) and pathological (dietary insult) conditions.

Hepatic FSP27 significantly suppresses mitochondrial FA β-oxidation and decreases TG turnover[44, 45]. Forced expression of hepatic FSP27 both *in vivo* and *in vitro* led to increase in LDs and TG levels[45], while knockdown of *Fsp27* improved hepatic steatosis[44-46], glucose tolerance[47] and even reduced atherosclerotic lesion in mice[48]. Furthermore, overexpression of FSP27 impairs ketogenesis[49] which may promote the development of hepatic steatosis and NAFLD[50].

In humans, there is a growing body of evidence that CIDEC is having role in the pathophysiology of hepatic steatosis[51-53].

1.1.1.4 *FA secretion*

In the liver, FAs are esterified together with glycerol to form TG, or with cholesterol to form cholesteryl esters (CE). Hence, FA not fated to oxidation are stored inside LDs as TG and CE or secreted in the core of very low-density lipoprotein (VLDL) particles. The VLDL secretion from the liver is a two-step process. In the first step, lipid-poor particles (pre-VLDL) are formed by the interaction between apolipoprotein B-100 (apoB-100) and the lipids of the membrane of the endoplasmic reticulum (ER)[54], through the action of microsomal triglyceride transfer protein (MTP)[55]. In the second step, further lipidation of pre-VLDL leads

to TG-rich mature VLDL fated to secretion[56]. Each VLDL particle has a single molecule of apoB-100. Hence, VLDL production and secretion depends mainly on apoB synthesis, MTP, insulin and on the availability of lipids[57]. ApoC-III and apoE are also important regulators of VLDL metabolism.

VLDL secretion provides a mechanism for reducing hepatic TG and CE levels. Impairment in hepatic VLDL secretion caused by genetic defects[58] or pharmacological inhibition of MTP[59] is associated with hepatic steatosis. However, patients with NAFLD have higher secretion rate of VLDL-TG than subjects with normal hepatic TG content[60] suggesting that the levels of lipid accumulation *per se* fuel VLDL secretion.

Most of the hepatic TG synthesized by DNL is stored in the cytoplasmic LD and only a smaller portion is secreted as VLDL[61]. Moreover, increased hepatic DNL is not linked with increased VLDL production in mice[62]. Recent studies have shown that liver-specific inhibition of DNL increased VLDL-TG secretion and reversed hepatic steatosis in mice and humans[15, 63]. DNL inhibition decreases polyunsaturated fatty acid (PUFA) levels that induce SREBP-1c, which in turn increases glycerol-3-phosphate acyltransferase 1 (GPAT1) expression and TG synthesis, and consequently VLDL secretion[63].

In healthy individuals, ~70% of FA incorporated into VLDL-TG are derived from circulating NEFA[2]. Increased NEFA release, as observed in insulin-resistant states, increases the availability of hepatic lipids and stimulates the production and secretion of VLDL particles[55].

One *in vitro* study using hamster hepatocytes suggested that increased CE levels to enhance VLDL secretion rate[64]; however, evidence of increased secretion of VLDL-TG was reported in a mouse model of low hepatic CE[65]. Nevertheless, the amount of CE in the ER seems to regulate the VLDL secretion, at least in part, by determining whether apoB-100 will be degraded or secreted[66].

1.1.1.4.1 Role of LD proteins in VLDL lipidation

As mentioned above, the availability and capability to transfer neutral lipids to pre-VLDL particles are essential for VLDL assembly and maturation. Neutral lipids are stored within LD in all cell types[67]. Impairment of LD proteins and subsequent inadequate lipidation may result in premature degradation of apoB-100.

CIDEB, a member of the CIDE family, is expressed mainly in the liver and kidneys. *Cideb-/-* mice have lower plasma levels of TG, enhanced hepatic FA oxidation, and are resistant to hepatic steatosis with improved insulin sensitivity[68]. Further analyses have shown that CIDEB is enriched at LDs and the ER where it interacts with apoB. Moreover, VLDL particles secreted from *Cideb-/-* mice displayed less TG content but similar number of apoB-100 particles, indicating an impairment in VLDL lipidation[69].

Perilipin 2 (Plin2) is a ubiquitously expressed LD associated protein[70] which enhances TG storage and reduces VLDL-TG secretion[71]. In *Cideb-/-* mice, knockdown of hepatic *Plin2*

resulted in improved hepatic steatosis with increased VLDL-TG secretion. These data uncover opposing roles of CIDEB and PLIN2 in controlling VLDL lipidation[72].

1.1.2 Mechanism of lipid-induced hepatic insulin resistance (IR)

In the liver, insulin binds to and stimulates insulin receptor tyrosine kinase (IRTK) that phosphorylates insulin receptor substrates (IRS2)[73]. Phosphorylation of IRS2 results in a cascade of different proteins phosphorylation, leading to recruitment of Akt2[74]. Phosphorylated Akt2 suppresses hepatic glucose production under insulin stimulation via two mechanisms: first, decreased gluconeogenesis and secondly, increased glycogenensis[75].

In a rat model of NAFLD, insulin ability to suppress hepatic glucose production is diminished[76]. Hepatic IR in this model is associated with high levels of hepatic diacylglycerol (DAG) and increased translocation of protein kinase-Cε (PKCε) towards plasma membrane where it suppresses IRS2 activity[75]. Furthermore, hepatic knockdown[77] or knockout of PKCε[78] protects from lipid-induced IR. Hepatic DAG content in cytoplasmic LDs is reported to be the best predictor of hepatic IR in obese, nondiabetic individuals[79]. Hepatic DAG level and PKCε activity were also strongly correlated in these individuals.

Several other mechanisms have been proposed to verify the link between hepatic lipid and IR (for review see[80]); however it seems that DAG-induced PKCε activation is still the most acceptable mechanism to explain the development of hepatic IR in different experimental and clinical models[75].

According to the DAG- PKCε hypothesis, only DAG in cytoplasmic LDs can be translocated to the plasma membrane to activate PKCε. The entrapment of TGs and its hydrolytic by-product DAG within the LDs prevents high-fat diet induced hepatic IR in CGI-58 knockdown mice[81, 82]. Surprisingly, this mouse model remains insulin-sensitive despite severe hepatic steatosis[81]. The compartmentation and prevention of TG hydrolysis inside LDs lead to impairment of DAG-mediated hepatic IR and dissociate hepatic steatosis from IR. These data may explain why not all animals or individuals who develop hepatic steatosis develop IR[82].

Taken together, the DAG- PKCε hypothesis uncovers the crucial potential role of regulation of lipid partitioning and trafficking by LDs proteins in the pathophysiology of lipid-induced hepatic IR.

1.1.3 NAFLD and IR

The development of hepatic steatosis during the pathogenesis of NAFLD is thought to be driven by IR, which increases the efflux of NEFAs to the liver from visceral fat stores and peripheral lipolysis[83]. Nevertheless NAFLD *per se* exacerbates hepatic IR and increases the risk of developing T2DM[75, 84]. Studies of NAFLD in animal models and humans have consistently demonstrated the presence of an underlying IR[85].

Although high calorie intake results in obesity, only those with hepatic steatosis will develop IR[75]. In line with this, postprandial glucose levels in lean IR individuals demonstrate that the

energy derived from ingested carbohydrates is shifted from muscles to the liver which promote hepatic steatosis; this suggest that skeletal muscle IR precedes hepatic IR which in turn may predispose them to NAFLD[86]. Furthermore, elevation of liver enzymes has been shown to predict the development of IR and T2DM[87, 88].

As hepatic steatosis and IR go hand in hand, therapies that improve IR also improve hepatic steatosis[28, 29, 89-91] and treatment of hepatic steatosis improves IR[8, 15].

As mentioned above, it has been demonstrated that the increased hepatic DAG associated with NAFLD activates PKC, resulting in deterioration of insulin signaling[84]. Hepatic IR is characterized by decreased repression of endogenous hepatic glucose synthesis and contributes to additional increased whole-body IR[4].

1.1.3.1 *Role of CIDEC/FSP27 in IR*

The consequences of complete loss of FSP27 on IR are controversial, as two independent studies showed that *Fsp27-/-* mice displayed improved insulin sensitivity and were resistance to diet-induced obesity[92, 93]. In contrast, a recent study showed that *Fsp27-/-* mice developed hepatic steatosis and IR[94]. Moreover, a lipodystrophic patient with IR diabetes has been identified carrying a homozygous nonsense mutation in CIDEC[95].

The adipocyte-specific disruption of FSP27 causes hepatic steatosis and IR in mice on a high-fat diet[96], suggesting that the impaired fat-storing function of adipocytes results in sustained delivery of NEFA from adipose tissue to the liver which leads to hepatic steatosis. However, partial silencing of FSP27 using antisense oligonucleotides (ASO) displayed improved insulin sensitivity and glycemic control in mice[47]. These discrepancies in outcomes between knockout and knockdown studies are probably due to the residual FSP27 activity in adipose tissue which prevents the sustained release of NEFA from adipose tissues and its deleterious consequences.

Taken together, these results suggest that only hepatic CIDEC/FSP27 should be targeted in order to protect from hepatic steatosis and IR.

1.1.3.2 *Role of inhibition of DNL in IR*

Inhibition of DNL in rats with diet-induced obesity improves hepatic steatosis and glucose-stimulated insulin secretion, and decreases hemoglobin A1c[97]. The insulin-sensitizing effects of metformin and other DNL inhibitors were discussed earlier (see section 1.1.1).

1.1.4 Genetic predisposition to NAFLD

Several lines of evidence indicate that NAFLD develops as a consequence of complex multifactorial processes among which genetic susceptibility and environmental factors are involved. Furthermore, the severity and progression of NAFLD are modulated by liver specific epigenetic and microRNA alterations that significantly affect the liver transcriptomic profile (for review see[98]).

The results from the first genome-wide association study (GWAS) of NAFLD have greatly increased the knowledge about the genetic component of the disease. It showed that genetic variation in patatin-like phospholipase domain containing 3 (PNPLA3) is significantly associated with hepatic steatosis, inflammation and consequently the individuals' susceptibility to NAFLD[99]. Many other NAFLD-GWAS studies uncovered different genetic variants. However, so far, missense variants in different loci have only been found with replicated evidence for the PNPLA3, transmembrane 6 superfamily member 2 (TM6SF2) and glucokinase regulatory gene (GCKR) genes[98].

1.1.5 NAFLD and metabolic syndrome

The MESA (Multi-Ethnic Study of Atherosclerosis) study reported higher prevalence of the metabolic syndrome in subjects with NAFLD compared to subjects without NAFLD[100]. In NAFLD patients, nine out of ten have more than one component of the metabolic syndrome and ~30% met all the criteria for diagnosis[101]. Moreover, many NAFLD patients die from cardiovascular disease (CVD), which is the major cause of death in subjects with metabolic syndrome, even more common than liver-related complications[102]. NAFLD also shares common pathophysiology with CVD, such as IR, oxidative stress, low-grade inflammation, and atherogenic dyslipidemia[100 103, 104].

Based on the above data, NAFLD represents the hepatic manifestation of the metabolic syndrome[6].

1.1.6 Treatment options specific for NAFLD

There is no drug that has a specific indication for NAFLD treatment. Current therapeutic options include intensive lifestyle modification (e.g. weight loss, exercise, diet and vitamin E supplementation)[105]. Many clinical trials have been performed based on the fact that NAFLD and the metabolic syndrome share fairly similar pathophysiology; thus pharmacological treatment of metabolic comorbidities could lead to improvement of liver histology in NAFLD subjects. However, none of these studies have demonstrated significant benefits[105]. Also, PUFAs have been reported to reduce IR, lipogenesis and systemic inflammation; however, in addition to negative side effects, no significant effects on hepatic steatosis or fibrosis were found in subjects treated with PUFAs[106].

In a recent meta-analysis, the effects of metformin on NAFLD were investigated. It was found that although treatment with metformin improved liver enzymes, it had no significant histological effects[107]. Treatments with lipid-lowering drugs such as statins or ezetimibe can be used to treat dyslipidemia, commonly observed in subjects with NAFLD. Treatment with atorvastatin reduces hepatic steatosis whereas ezetimibe improves hepatic histology (for review see[108]). However, lipid-lowering drugs are currently not labelled for the treatment of NAFLD/NASH, due to insufficient evidence to recommend the use of these drugs[4].

At the moment, liver transplantation could be the only option for end stage patients. Although it can be successful, liver transplantation is dependent on the availability of organs, carries significant risks[105] and is also expensive.

Thus, new therapeutic strategies to tackle the lipid accumulation, improve IR and prevent the disastrous consequences of NAFLD are needed.

1.2 LIVER AND CHOLESTEROL

The liver has a key role in the whole-body cholesterol hemostasis. Almost all cells in the body can synthesize cholesterol; however the main *de novo* cholesterol synthesizing organ is the liver. Moreover, it handles the dietary cholesterol absorbed through the intestine and it is also responsible for cholesterol disposal into the bile.

Cholesterol is known to have a number of biological functions being an essential component of cell membrane, bile acids (BAs) and steroid hormones precursor, and to function as a regulator of transcriptional responses. In the skin cholesterol is the precursor of vitamin D. The majority of cholesterol exists in free (unesterified) form in the cell membrane where it determines the degree of membrane fluidity and consequently the kinetic of anchored proteins.

To prevent the cytotoxic free cholesterol (FC) accumulation, cholesterol is converted to CE by covalently attaching the sterol moiety with long chain FA, and stored in the cytoplasmic LDs. Cholesterol esterification reactions are catalyzed by three enzymes: i) lecithin-cholesterol acyltransferase (LCAT); ii) acyl-coenzyme A:cholesterol acyltransferase (ACAT) 1, and iii) ACAT2. LCAT acts solely in plasma and esterifies the lipoprotein-associated cholesterol whereas ACAT1 and ACAT2 are both involved in intracellular cholesterol esterification[109].

1.2.1 Cholesterol hemostasis

Cholesterol homeostasis is strictly regulated and it is maintained by the balance between cholesterol synthesis (endogenous pathway), cholesterol absorption (exogenous pathway), biliary cholesterol excretion and transintestinal cholesterol excretion (TICE).

1.2.1.1 *Endogenous pathway*

Cholesterol biosynthesis starts with the condensation of one molecule of acetyl-CoA with one molecule of acetoacetyl-CoA to produce 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) by HMG-CoA synthase (HMGCS). HMG-CoA is reduced to mevalonate by the rate-limiting enzyme HMG-CoA reductase (HMGCR), anchored in the membrane of the ER[110]. Then the condensation reactions continue until the formation of the 27-carbon-containing cholesterol molecule. Liver and intestine account for most cholesterol synthesis though most cells in the body can synthesize cholesterol. Statins are the most prescribed group of plasma lipid lowering drugs and act through inhibition of HMGCR.

The liver has a key role in the regulation of the whole-body cholesterol homeostasis[111]. As mentioned above, in the hepatocyte cholesterol is transported to the ER and esterified with ACAT2. CEs can then either be stored inside LDs or packed with apoB-100 and other lipids and secreted in the circulation as nascent VLDL. After the secretion, VLDL particles acquire apoE and and apoC-II[111]. VLDL particles are rapidly removed from the circulation by extra-hepatic tissues through VLDL receptor (VLDLR), which binds apoE in FA-active tissues [112]. In these tissues, lipoprotein lipase (LPL) is upregulated in the fed states, which hydrolyzes VLDL-TG into glycerol and NEFA. LPL is expressed mainly in the capillary endothelial surface of the adipose, muscle and heart tissue and is activated by apoC-II[113]. NEFAs taken up by the tissues can either be used for energy production (skeletal and cardiac muscles), storage (adipose) or be transported in the circulation together with albumin. The reduction of TG content changes the size and the density of VLDL, converting these particles into intermediate density lipoprotein (IDL). The TG carried by IDL are further hydrolyzed by hepatic lipase which converts IDL particles into TG-poor and cholesterol-rich low density lipoprotein (LDL) particles[114].

The lipid content and composition of lipoprotein particles are also affected by plasma lipid transfer proteins, which include the cholesteryl ester transfer protein (CETP) and the phospholipid transfer protein (PLTP). CETP facilitates the removal of high density lipoprotein (HDL)-CE in exchange for TG carried within VLDL or LDL, whereas PLTP transfers PL from TG-rich particles to HDL[115]. IDL and LDL particles are taken up from the circulation mainly via the hepatic LDL receptor (LDLR); however, other members of the LDLR family of proteins are involved in this process such as the LDLR-related protein 1 (LRP1)[116].

PL and free cholesterol can be secreted to stabilize lipid poor apoA-1 by the adenosine triphosphate (ATP)-binding cassette transporter A1 (ABCA1), which is expressed on hepatocytes, enterocytes and macrophages. This process is fundamental for HDL formation, account for the cholesterol removal from peripheral tissues towards the liver, and is called reverse cholesterol transport (RCT) pathway[117]. The liver takes up HDL via the scavenger receptor class B type I (SR-BI).

1.2.1.2 *Exogenous pathway*

The adult human body contains around 140 g of cholesterol of which less than 1% is lost per day. The body is fully capable of *de novo* synthesizing all the cholesterol required for its biological processes. Daily, 1200 to 1700 mg of cholesterol enters the small intestine, of which only 300 to 500 mg is of dietary origin; the rest comes from cholesterol excreted in bile and a small part from the intestinal mucosal turnover[118]. The dietary CE requires emulsification with BAs to form micelles that increase its exposure to hydrolysis before the micelles are taken up by the enterocytes. In the small intestine, cholesterol absorption is facilitated by the Niemann-Pick C1-like 1 protein (NPC1L1)[119]. Ezetimibe was first developed as a potential inhibitor of ACAT, but was instead found to inhibit intestinal

cholesterol absorption by binding NPC1L1[120, 121]. NPC1L1 is also involved in the absorption of plant sterols.

ABC transporters G5 (ABCG5) and G8 (ABCG8) act as obligatory heterodimers and are expressed at the apical membrane of enterocytes, where they pump FC back to the intestinal lumen[122]. ABCG8 are also expressed in the hepatocyte apical membrane where they excrete sterol into the bile[123]. Overexpression of both hepatic and intestinal isoforms of ABCG5 and ABCG8 decreased the intestinal cholesterol absorption by ~50%, and increased biliary cholesterol levels five times more in mice[122].

Both endogenous and exogenous free cholesterol are esterified by ACAT2 in enterocytes. CE and TG are incorporated together with apoB-48 into chylomicrons which are secreted to the lymphatic system then passed to circulation through the thoracic duct. Chylomicrons have a similar fate as VLDL: they acquire apoE and apoC-II and their TG content is hydrolyzed by LPL in the bloodstream[124]. Chylomicrons also can bind to VLDLR and exchange lipids and apolipoproteins with other lipoprotein particles. These modifications result in the formation of chylomicron remnant particles, rich in apoE and cholesterol. Chylomicron remnants are rapidly removed from the circulation via LDLR or LRP1. Chylomicron CE is removed almost exclusively by the liver, whereas the liver accounts for only 20-30% of chylomicron TG removal from circulation[125].

1.2.1.3 *Cholesterol excretion*

The fecal excretion of cholesterol is the major way for its disposal since it cannot be catabolized. The liver excretes cholesterol into the bile as such via ABCG5:ABCG8 heterodimers[126] or after its conversion into more soluble BAs. Excess biliary cholesterol secretion is considered as the main determinant of cholesterol gallstone formation[126]. The rate-limiting enzymes converting cholesterol to BAs are cholesterol 7α-hydroxylase (CYP7A1) and sterol 27-hydroxylase (CYP27A1)[127]. CYP7A1 regulates the classical or neutral pathway, whereas CYP27A1 mediates the alternative or acidic pathway. BAs are secreted from the liver into the bile via ABC transporter B11 (ABCB11), whereas PLs are secreted via ABC transporter B4 (ABCB4). In contrast to rodents, humans express NPC1L1 on bile canaliculi; however its role in the regulation of cholesterol metabolism and whether it can be inhibited by ezetimibe has not been fully elucidated (for review see[128]). Cholesterol excretion in the feces by a non-biliary pathway is observed in animal models and known as TICE. In humans, the presence and the extent of TICE still needs further investigations (for review see[129]).

1.2.1.4 *Regulation of cholesterol hemostasis*

Through tight regulation of HMGCR at transcriptional and post-transcriptional levels, intracellular cholesterol homeostasis is maintained[130]. The by-products of cholesterol synthesis pathway (e.g. mevalonate and isoprenoids) exert negative feedback inhibition on HMGCR, balancing cholesterol synthesis with cellular needs[131].

The intracellular levels of FC and oxysterols regulate the HMGCR turnover through the SREBP2 system. Newly synthesized SREBP2 binds to SREBP cleavage-activating protein (SCAP) in the ER. A decrease in sterol levels in the ER result in translocation of the SCAP-SREBP2 complex to the Golgi, where SREBP2 is processed to release a transcriptionally active N-terminal domain. After migration to the nucleus, the domain binds the sterol regulatory element (SRE) of target genes (e.g. *HMGCR*, *LDLR*, and *SREBF2*) and activates the transcription. Conversely, a little rise in the sterol levels in the ER results in retention of the SCAP-SREBP2 complex, as a consequence of an interaction between SCAP, sterols and insulin induced gene (INSIG). High levels of lanosterol (an intermediate in the synthesis of cholesterol) stimulate the binding of HMGCR to INSIG. This binding mediates the ubiquitination and subsequent degradation of HMGCR[132].

AMPK is a sensor of cellular energy status, and AMPK is stimulated when the AMP:ATP ratio is increased (indicating a low energy status). The activated AMPK phosphorylates and inhibits HMGCR activity since cholesterol synthesis is a highly energy consuming process. Protein phosphatase 2A dephosphorylates HMGCR, reactivating the enzyme[130].

Recently, a novel mechanism involving the miR-33a was identified. This microRNA was found to be co-transcribed within an intron of the primary transcript of SREBP2[133] and to mediate *ABCA1* and *ABCG1* mRNA degradation. Thus, low levels of intracellular sterols stimulate the expression of genes account for cholesterol synthesis and uptake (via SREBP2) and simultaneously reduce the levels of genes involve in cholesterol efflux (via miR-33a).

Furthermore, oxysterols play a direct role in the regulation of cholesterol homeostasis. Compared to cholesterol, oxysterols have very low concentrations in mammalian systems; however, in tissues where oxysterols to cholesterol ratio exceeds 1:1000 (e.g. brain and cholesterol-loaded macrophages), oxysterols may activate the liver X receptor (LXR)[134]. LXR is a nuclear receptor able to induce genes involved in the excretion of cholesterol. Accumulation of cholesterol is thought to increase the production of oxysterols and consequently activation of LXR and the LXR-target genes (ABCA1, ABCG1, ABCG5, ABCG8, and CYP7A1). This may represent a defense mechanism counteracting intracellular accumulation of cholesterol by increasing cholesterol efflux in addition to bile acid synthesis[134]. Oxysterols have also been shown to limit intracellular cholesterol levels through the INSIG-SREBP2-HMGCR system *in vitro*[134].

1.2.2 Simvastatin and ezetimibe combination therapy

2016 ESC/EAS Guidelines for the Management of Dyslipidaemias recommends statin as a first-line therapy, both in secondary and primary prevention[135]. Adverse muscle effects are fairly common with statins[136], and often only lower, and thus less effective, doses are tolerated. Inhibition of the intrahepatic cholesterol synthesis is compensated by an upregulation of LDLR, which reduces both plasma LDL cholesterol (LDL-C) levels and ASCVD[137]. Simvastatin and pravastatin are less effective compared to atorvastatin and rosuvastatin. All of them are generic and very cheap in Sweden. Simvastatin has for long

been the most used statin in Sweden and it decreases plasma LDL-C levels between 30-50%[137].

Ezetimibe inhibits intestinal cholesterol absorption by blocking NPC1L1[120, 121]. When given as monotherapy ezetimibe lowers plasma LDL-C levels by approximately 20 %, mainly by increasing LDL catabolism[138]. NPC1L1 is highly expressed in the human, but not in the mouse liver[128]. Studies in transgenic mice overexpressing human NPC1L1 in hepatocytes suggested that ezetimibe may also inhibit hepatic NPC1L1[139]. However, no mechanistic study regarding hepatic NPC1L1 in humans has been presented so far[128].

The hepatic LDLR expression was more efficiently upregulated with combined treatment than with monotherapy in pigs[140]. Moreover, kinetic studies in humans have revealed that the combination treatment of ezetimibe and simvastatin more effectively reduces apoB-48/apoB-100 containing lipoproteins than monotherapies[141]. Synergistic effects of adding ezetimibe to simvastatin has been demonstrated *in vivo* (for review see[142]).

The ENHANCE (The Ezetimibe and Simvastatin in Hypercholesterolemia Enhances Atherosclerosis Regression) trial in patients with familial hypercholesterolemia reported significant additional plasma LDL-C-lowering by adding ezetimibe to simvastatin (~ 18%), but without additional clinical effects compared to simvastatin alone[143].

The first evidence that adding ezetimibe to simvastatin improves the clinical outcome came in 2011 when the SHARP (Study of Heart and Renal Protection) study was published showing that the combination therapy reduced major atherosclerotic events by 17%[144]. However, the study did not include simvastatin as monotherapy. On the other hand, at least two well controlled studies with statins in the same kind of patients had no positive effect on the predefined primary end-points[145, 146].

As lines of evidence accumulated, NICE guidance recommended ezetimibe as adjuvant therapy for people with primary hypercholesterolemia[147].

Most recently, The Improved Reduction of Outcomes: Vytorin Efficacy International Trial (IMPROVE-IT) gives better study design by comparing ezetimibe-simvastatin combined therapy against simvastatin monotherapy. During the study, patients with recent acute coronary syndrome were recruited and regularly followed-up over 6 years. The overall benefit of combination therapy compared to simvastatin monotherapy was a 2% absolute risk reduction of cardiovascular events after 7 years with the number needed to treat of 50. Moreover, no additional side effects were reported by adding ezetimibe[148]. Interestingly, the beneficial effect was more pronounced in the subgroup of patients with T2DM in which a 5.5% absolute risk reduction was observed[149].

1.2.3 ACAT1 and 2

As mentioned above, the CEs synthesis is catalyzed by three enzymes: LCAT which acts exclusively in the circulation where it preferentially uses the FA present in position sn-2 of

the phospholipid present on the surface of lipoproteins (often linoleic acid-18:2), and ACAT1 and ACAT2, which both act intracellularly and preferentially use oleic (18:1) and palmitic (16:1) acids as substrates for cholesterol esterification[109, 150].

1.2.3.1 *Discovery of the two ACAT isoforms*

The ACAT-mediated esterification of cholesterol with palmitic acid in rat liver homogenates was first demonstrated in 1957[151], and for many years researchers thought that only one isoform was mediating the intracellular cholesterol esterification. It took three decades before the discovery of different rates of inhibition of ACAT activity in various tissues[152], suggesting other ACAT isoforms may exist. After five years, Chang and colleagues identified the DNA sequence of the ACAT1gene[153] . Thereafter, it became clear that at least two cholesterol esterifying enzymes may exist intracellularly when studies revealed close to normal hepatic and intestinal cholesterol esterification rates despite successful disruption of the *Acat1* gene[154].

1.2.3.2 *Function and activity of the two ACAT isoforms*

ACAT1 and ACAT2 are integral membrane proteins in the rough ER[155]. ACAT1 is found in most cell types whereas ACAT2 is exclusively expressed in hepatocytes and enterocytes[156, 157]. The ACAT1 and ACAT2 enzymes are encoded by sterol O-acyltransferase 1 and 2 (*SOAT1* and *SOAT2*), respectively[158]. Humans have higher intestinal than hepatic ACAT2 activity (40-fold)[159], whereas mice and non-human primates have similar ACAT2-activity in enterocytes and hepatocytes. Sex-related differences have been reported in Chinese gallstone-patients with women having about 70% lower hepatic ACAT2 activity compared to men[160]. The opposite sex-related difference is found in mice (Figure 1). Since ACAT2 activity promotes atherogenesis (see below), the sex-related differences in humans and in mice can in part explain why women have delayed atherosclerosis while in female mice it is accelerated[161].

The wide tissue distribution of ACAT1 suggests that it functions to maintain the level of FC concentrations for optimal membrane function[162] and below toxic levels[109].The restricted expression of ACAT2 to the major apoB-containing lipoprotein producing cells (hepatocytes and enterocytes) suggests a more specialized role in packaging CE into VLDL and chylomicrons, respectively. In addition to these functions, inverse relationships between dietary cholesterol and cholesterol absorption have been observed in *Soat2-/-* mice, which emphasized the importance of intestinal ACAT2 activity for cholesterol absorption[158, 163]. *Soat2-/-* mice maintained normal hepatic cholesterol concentrations irrespectively of the amount of cholesterol in the diet[163]. These studies also confirmed that the ACAT2 deficiency does not result in an upregulation of hepatic or intestinal ACAT1[163].

Moreover, Alger *et al*[65] reported that inhibition of hepatic ACAT2 in mice reduces dietary cholesterol-induced hepatic steatosis. The authors suggested that hepatic CE accumulation

hinders TG export through VLDL and consequently lead to hepatic steatosis in mice fed cholesterol-enriched diets.

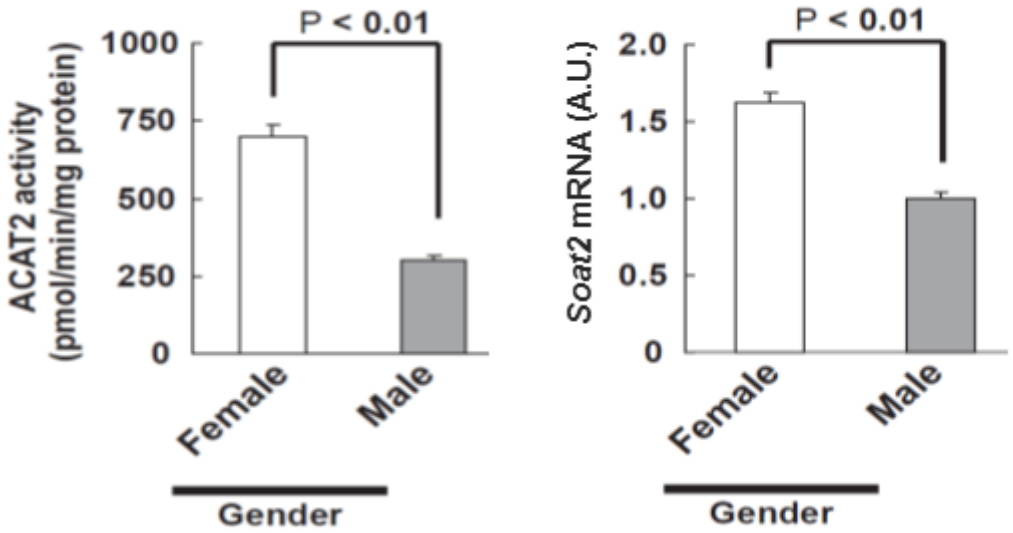

Figure 1. Sex-related differences in ACAT2 activity and *Soat2* mRNA levels in mice (Parini P *et al*, unpublished).

1.2.3.3 *Regulation of ACAT2*

1.2.3.3.1 *Soat2* is transcriptionally regulated by cholesterol

SREs are present within the promoters of many cholesterol-regulated genes. However, as no SREs have been identified within the human *SOAT1*[164, 165] or *SOAT2* promoters[166] they were not thought to be transcriptionally regulated by cholesterol. The first observation regarding a potential transcriptional regulation by cholesterol came from studies in monkeys where high dietary cholesterol resulted in increased hepatic *SOAT2* expression[167]. Later, Parini P *et al.* showed that treatment with high-dose atorvastatin significantly reduce *SOAT2* mRNA and protein levels and enzymatic activity in humans[168]. In 2007 our group showed that treatment of human hepatic Huh7 and HepG2 cells with cholesterol increased *SOAT2* mRNA levels and enzymatic activity, further supporting a transcriptional regulation of *SOAT2* by cholesterol also in humans[169].

1.2.3.3.2 Transcription factors involved in the regulation of SOAT2

We have identified an important hepatocyte nuclear factor 1 (HNF1) binding site in the human *SOAT2* promoter that functions as a positive regulator sequence. Both HNF 1α and HNF1β can bind to this sequence and control hepatic *SOAT2* expression[170]. We have also identified HNF4 α, an upstream regulator of HNF1α, to be an important positive regulator of the human hepatic *SOAT2*gene[171]. Caudal-related homeodomain protein can bind to mouse and human *SOAT2* promoter regions[172] and together with HNF1 α act to positively regulate the intestinal *SOAT2* expression[173]. Recently we identified TG-interacting factor 1 to be a

transcriptional repressor of the human *SOAT2* gene, which could block *SOAT2* induction by HNF 1α and HNF4α[174].

1.2.3.4 *Protective Role of ACAT2 depletion*

In addition to protection against atherosclerosis, ACAT2 depletion in mice has other beneficial effects as it protect against diet-induced hypercholesterolemia, cholesterol gallstone disease as well as dietary cholesterol-induced hepatic steatosis.

1.2.3.4.1 Preclinical studies

Mice

The involvement of ACAT2 in the pathogenesis of atherosclerosis was first studied in *ApoE/Soat2* double knockout mice[175]. Total serum cholesterol was decreased in these mice compared to *Apoe-/-* mice, principally due to a more than 70% reduction in serum CE. Female *ApoE/Soat2* double knockout mice on chow diet had significantly decreased levels of aortic atherosclerosis compared to controls[175]. Similarly, *Ldlr/Soat2* double knockout mice fed a high-fat diet showed greatly reduced aortic atherosclerosis (> 80%) compared to controls[176]. These studies suggested ACAT2-derived CE in apoB-containing lipoproteins to be the most atherogenic lipid in the circulation.

Intestinal specific LXR activation protects from atherosclerosis by stimulation of the RCT and decreases circulating CE in apoB-lipoproteins through reducing both hepatic and intestinal ACAT2 activity[177]. Moreover, *Soat2-/-* mice fed diets with different FAs composition were protected from atherosclerosis regardless of the type of fat[178]. Furthermore, selective inhibition of ACAT2 has been consistently shown to be atheroprotective in different mouse models without causing negative effects[158, 175, 179-181].

Liver-specific inhibition of ACAT2 may provide more clinical benefit for atherosclerosis prevention than intestinal ACAT2 inhibition[179, 182].

Monkeys

An association between the biosynthesis and secretion of hepatic ACAT-derived cholesteryl oleate has been shown in African green monkeys fed high dietary cholesterol. Also, the hepatic ACAT activity was found to be correlated with LDL particle size. Larger LDL was enriched primarily with cholesteryl oleate and appeared to promote coronary artery atherosclerosis as proved by the strong correlation with increased coronary artery intimal thickness[183].

Consistently, increased concentrations of plasma cholesteryl oleate were proportional to an increase in LDL particle size and coronary atherosclerosis levels[150, 184, 185].

The above data support the hypothesis that ACAT2-derived CE makes LDL particles larger than normal and more prone to bind to arterial proteoglycans, which increase CE arterial uptake and foam-cell formation, early signs of atherosclerosis[186].

1.2.3.4.2 Human studies

As mentioned above, both ACATs preferentially use oleic and palmitic acids as FA substrates[150, 187], whereas, LCAT uses linoleic acid[188]. Plasma levels of linoleic acid were lower in patients with myocardial infarction than in controls[189, 190].

In the Uppsala Longitudinal Study of Adult Men (ULSAM), increased levels of palmitic and oleic acid predicted ASCVD events, whereas the proportion of linoleic acid was inversely related to mortality[191]. Patients with ASCVD had increased levels of oleic acids in the aorta[192].

The Atherosclerosis Risk in Communities (ARIC) study came to the same conclusions as it found average carotid intima-media thickness to be positively correlated with saturated and monounsaturated FA content, and inversely associated with PUFA level in CE[193].

Furthermore, plasma levels of ACAT2-derived CE strongly predict the probability of having coronary artery disease in a clinical setting[194].

Collectively, these data suggested ACAT2 as pro-atherogenic enzyme also in humans and its inhibition as an attractive target to protect against ASCVD through modulation of intestinal and hepatic cholesterol metabolism[195].

1.2.3.5 *ACAT2 as future target to treat hepatic steatosis*

As mentioned above, accumulation of CE within hepatocytes limit the mobilization of hepatic TG and lead to retention of CE and TG within hepatic LDs. Liver histology from wild type mice fed high cholesterol diets showed large LDs within most hepatocytes, whereas *Soat2-/-* had fewer and smaller LDs[65]. It has also been shown that administration of ASOs against ACAT2 reversed pre-existing hepatic steatosis in mice[65].

Nevertheless, all these studies have been performed in high cholesterol diet-induced hepatic steatosis models and whether ACAT2 inhibition *per se* could protect against hepatic steatosis independent of dietary regimens is not known yet.

1.2.3.6 *ACAT2 inhibitors*

Again, specific ACAT2 inhibition is considered an attractive and promising therapeutic target for the prevention and treatment of atherosclerosis and fatty liver[180, 181]. In contrast, unspecific ACAT inhibition has failed to show beneficial effects[196, 197]. This outcome was expected knowing that ACAT1 inhibition worsen atherosclerosis (for review see[195]) and the approach to use molecules that inhibit both ACAT1 and ACAT2 hardly criticized[198].

1.2.3.7 *Statin and ezetimibe effects on ACAT2*

As mentioned above, patients treated with 80 mg atorvastatin daily for four weeks had about 50% lower hepatic ACAT2 activity, protein and mRNA levels[168]. It thus seems that part of the beneficial effects of statins in lowering plasma cholesterol levels are due to reduced

hepatic ACAT2 activity and this further support inhibition of ACAT2 in the treatment and prevention of atherosclerosis[168]. Moreover, ezetimibe treatment reduced the expression levels of intestinal ACAT2 in animal models[199].

In *Apoe -/-*mice, ezetimibe was equally effective as selective ACAT2 inhibitors in reducing the atherosclerotic lesion areas of the aortae in a dose-dependent fashion whereas atorvastatin had no effect[181]. However, this discrepancy between the effect of ezetimibe and atorvastatin on atherosclerotic lesion is due to the animal model rather than their effect on ACAT2. Moreover, ezetimibe effectively reduced CE content of atherosclerotic lesion through reducing cholesterol absorption and possibly modulating ACAT2 activity.

1.2.4 Remnant-cholesterol

The remnant-cholesterol in the circulation consists of FC and CE carried within TG-rich lipoproteins including chylomicron remnants, VLDL and IDL. All these particles contain apoB as their main apolipoprotein component. The elevation of TG-rich particles is characteristic of T2DM dyslipidemia[200]. Remnant-cholesterol is estimated by subtracting LDL-C and HDL-C from total plasma cholesterol[201]. Since ACAT2 determines the cholesterol content of nascent VLDL and chylomicrons, it is therefore a major contributor to remnant-cholesterol in circulation.

Both elevated remnant-cholesterol and LDL-C increase the risk for ASCVD. However, large genetic study has recently recognized elevated remnant-cholesterol and not LDL-C to also be associated with systemic low-grade inflammation[201]. Thus, the residual risk of ASCVD seen after optimal lowering of LDL-C can partly be due to elevated remnant-cholesterol[202]. Remnant-cholesterol containing particles could be deposited in the arterial intima through the interaction between apoB-100 and arterial proteoglycans[203]. Hence, the ability of a lipid lowering drugs to decrease all apoB-containing particles that can retain FC and CE into the arterial walls could be equally important as its ability to lower LDL-C[202, 203].

According to the European Atherosclerosis Society (EAS) recommendations, the non-fasting lipid profile including calculation of remnant-cholesterol can be used routinely in clinics. Fasting remnant-cholesterol $\geq$ 0.8 mmol/L and non-fasting remnant-cholesterol $\geq$ 0.9 mmol/L are both considered to be abnormal[204].

1.2.5 Lipoproteins binding to arterial proteoglycans (PG)

Plasma circulating cholesterol-rich apoB-containing lipoproteins bind to human arterial proteoglycan (PG). This binding leads to lipoprotein contained cholesterol deposition in the intima of the arterial wall which is consider as an initial step in the atherogenesis process and is a fundamental concept in "response to retention hypothesis"[203, 205].

The LDL particles from ASCVD patients were found to have higher affinity for arterial PG compared to healthy[206]. Remnant particles have the ability to bind PG through their apoB-

100. Moreover, sdLDL particles which characterized T2DM with high ASCVD risk, have a higher binding affinity to arterial PG than other LDL subclasses[207].

Statin significantly reduced the affinity of LDL particles to arterial PG in patients with hypercholesterolemia[208].

2 AIMS AND SIGNIFICANCE

The overall aim of this thesis was to explore how the genetic and pharmacological interventions on hepatic and intestinal cholesterol homeostasis could affect the pathophysiology of hepatic steatosis, glucose tolerance, and insulin sensitivity as well as the potential impact on ASCVD.

The specific aims in the individual papers were:

Papers I and **II**

To elucidate the molecular mechanisms of *Soat2* depletion in the pathophysiology of hepatic steatosis and insulin sensitivity, independently of dietary regimens.

Paper III

To investigate the potential positive effects of cholesterol absorption and/or synthesis inhibition particularly in lipid disorders characterized by elevated remnant-cholesterol such as T2DM.

Paper IV

To characterize the effects of adding ezetimibe to simvastatin therapy in hepatic transcriptional signature to uncover the potential beneficial responses on different metabolic pathways in humans.

3 METHODOLOGY

The results presented in this thesis are generated using a wide range of methods spanning from basic molecular techniques to applications of highly sophisticated computational biology tools. In this section, the main methods are briefly described. A more comprehensive description of each experimental model and method are found in the papers which this thesis is based upon.

3.1 EXPERIMENTAL MODELS

3.1.1 *Soat2-/-* mice

The *Soat2-/-* mice were originally generated by Robert V. Farese Jr, CA, US[158]. Wild type (WT) mice were maintained by heterozygote breeding of mice on a mixed genetic background (C57BL6/SV129) where the C57BL6 represents > 90% of the background. Female and male mice, 9-15 weeks of age, were fed high-fat (HFD) or chow diets for eight weeks or a high-carbohydrate diet (HCD) for two and a half weeks. The mice were housed with their siblings in an enriched environment, and kept in the same room with controlled temperature and humidity, with free access to water and food, and maintained on a 12/12 hour light-dark cycle at the animal facility at Karolinska Institute (**Papers I** and **II**).

3.1.2 Human subjects

Forty patients (14 males, 13 fertile and 13 post-menopausal females) with uncomplicated cholesterol gallstone disease, eligible for elective cholecystectomy at the Department of Surgery, Danderyd Hospital, Danderyd, Sweden, were enrolled in the Stockholm study (**Papers III** and **IV**). Briefly, liver biopsies and bile were collected from the patients, following an overnight fast, after four weeks of treatment with simvastatin 80 mg daily (S), ezetimibe 10 mg daily (E), simvastatin 80 mg and ezetimibe 10 mg daily (S+E), or placebo (P). Blood samples were also collected before and after the treatments after an overnight fast. The patient selection, the inclusion and exclusion criteria were described in details in **Paper III**.

3.2 METHODS

3.2.1 Tissue lipids

Mouse liver sections were stained with Oil Red O to visualize lipids. Also, mouse liver and muscle lipids were extracted and quantified as describe in **Papers I** and **II**.

3.2.2 Quantification of lipolytic activity in adipose tissue

The lipolytic activity was assessed in gonadal adipose tissues from the mice. The assay was performed by modification of the method described by Dube *et al*[209]. The total lipolytic activities were measured by the enzyme fluorescence method using a LPL activity assay kit (**Paper I**).

3.2.3 RNA isolation, cDNA synthesis and real-time RT-PCR

Total RNA was isolated using Trizol (**Papers I** and **II**) or AllPrep DNA/RNA/protein kit (**Papers III** and **IV)** and transcribed into cDNA using Omniscript (Qiagen). The mRNA levels were quantified using SYBRGreen Mastermix and specific primers. Arbitrary units were calculated by linearization of the CT values and normalized to glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) for liver tissue, cyclophilin (*Ppia*) for adipose tissue (**Papers I** and **II**), and *GAPDH* for human liver tissue (**Papers III** and **IV)**.

3.2.4 RNA libraries preparation and sequencing

Extracted RNA for each sample was quantified and the integrity was verified using the Agilent Bioanalyzer. Samples with RNA integrity number (RIN) values above 7.5 were selected for RNA libraries preparation.

RNA libraries were prepared according to the Illumina TruSeq Stranded mRNA Sample Preparation protocol (Illumina, San Diego, CA). Briefly, mRNA was isolated from total RNA using the poly-A selection method. The isolated mRNA was fragmented using divalent cations and elevated temperature (94°C for 8 min). The first cDNA strand was then synthetized with reverse transcriptase and random primers while the second strand was synthesized with DNA polymerase I and RNase H. DNA products were then purified and those with adapter molecules on both ends were the only enriched with PCR. The mRNA libraries were validated using the Bioanalyzer, pooled and sequenced using Illumina NovaSeq 6000, S1 flow cell, PE 2x50bp sequencing system at SciLifeLab, Stockholm, Sweden (**Paper IV)**.

3.2.5 Western blot

Whole-cell, cytosolic and membrane proteins were prepared from each individual and equal amounts of protein from individuals in each group of mice were pooled. Three different concentrations of each protein were loaded and separated by electrophoresis, transferred to nitrocellulose membranes, blocked, and incubated with antibodies against CIDEC/FSP27, PLIN2 and GLUT2. Bands were visualized using Odyssey CLx (LI-COR, Lincoln, NE) and quantified by the image J software. CIDEC/FSP27, PLIN2 and GLUT2 levels were quantified by calculating the first derivative of the linear regression curve representing the three titration points for each sample pool. The slopes for the WT mice of respective diet were set to 1 (**Papers I** and **II**).

3.2.6 Serum analyses in mice

Serum lipoproteins were separated by size-exclusion chromatography (SEC) followed by on-line determination of total- and free cholesterol and TG[210]. Serum insulin, c-peptide, and glucose were measured using enzyme-linked immunosorbent assays (ELISAs) according to the manufacturer's protocols. Serum NEFA levels were measured using an enzymatic colorimetric assay (**Papers I** and **II**).

3.2.7 Insulin and oral glucose tolerance tests

Mice were intraperitoneally injected with 0.75 U/kg insulin or gavaged with 1 g/kg glucose after a four hour fast. Glucose concentrations were monitored at baseline and after 15, 30, 60, and 120 minutes (**Paper II**).

3.2.8 Human plasma and bile analysis

3.2.8.1 *General analyses*

C-reactive protein (CRP), creatine kinase (CK), apoE and safety analyses in addition to biliary total BAs were performed by certified routine assays at the Karolinska University Laboratory, Stockholm, Sweden. ApoC-III was analyzed using an ELISA kit according to manufacturer's instructions **(Paper III).**

3.2.8.2 *Lipoprotein profiling*

In addition to the SEC performed as above, detailed analyzes of lipoprotein subclasses and apoA-1 and apoB were performed with Nuclear Magnetic Resonance spectroscopy (NMR) at Nightingale Health Ltd (formerly known as Brainshake) Laboratory, Vantaa, Finland[211] **(Paper III)**.

3.2.8.3 *Plasma and biliary lipids*

Plasma and biliary lathosterol and campesterol levels were determined by isotope dilution-mass spectrometry (MS)[212, 213]. The lathosterol/cholesterol and campesterol /cholesterol ratios were calculated and used as biomarkers for the whole-body cholesterol synthesis[214] and the intestinal cholesterol absorption[215], respectively. The plasma level of the BA synthesis intermediate, 7α-hydroxy-4-cholesten-3-one (C4) was analyzed by LC-MS/MS[216]. Biliary lipids were extracted using the Bligh-Dyer method[217] and cholesterol and PL levels were quantified by enzymatic methods whereas individual BAs were analyzed using GC-MS. The cholesterol saturation index was calculated using the critical tables described by Carey[218] (**Paper III**).

3.2.8.4 *Proteoglycan (PG) binding*

Solid phase binding assay was used, which has been found reproducible for evaluation of affinity of isolated, or in plasma, apoB-lipoproteins for human arterial PGs or purified PGs preparations[219, 220] (**Paper III**).

3.2.9 Bioinformatics Methods

3.2.9.1 *Mapping to human genome*

The RNA sequencing reads were mapped to the human genome version GRCh38[221] using STAR mapper[222] version 2.5.3a. Gene quantification was performed using the quantMode GeneCounts option during the STAR run (**Paper IV**).

3.2.9.2 *Quality control (QC)*

We normalized our reads to the library size and then ran principal component analysis (PCA) to assess the homogeneity of the transcriptomic data. The outlier samples shown by the PCA plot were then excluded from further analyses (**Paper IV**).

3.2.9.3 *Differential expression (DE) analysis*

To infer differentially expressed genes from our data, we used DESeq2 package within R programming language[223]. Briefly, filtering of zero counts was performed, followed by geometric normalization of all samples. Logarithmic-fold change (LFC) was used to estimate the effect size. To improve this estimator we shrunk the LFC and used the Approximate Posterior Estimation for global linear models (apeglm) method[224] (**Paper IV**).

3.2.9.4 *Pathways and gene ontology (GO)*

REACTOME pathways and GOs analyses were performed using the standard web tool ToppFun (https://toppgene.cchmc.org) (**Paper IV**).

3.2.10 Statistical analysis

All data are presented as mean ± standard error of the mean (SEM) unless otherwise stated. Data were log transformed prior to the parametric analysis when homoscedasticity was not present (unequal variance of the different groups). Outlier analysis was performed for all variables and rejection was done if outliers were present.

The relative reduction of LDL-C (one of the primary endpoints in the Stockholm study) was used as the variable on which sample size and power calculations were performed (**Papers III** and **IV**). Considering an LDL-C lowering effect of 15%, 30% and 50% by treatment with ezetimibe, simvastatin and ezetimibe plus simvastatin respectively, the calculated power was 0.8 if there were 10 patients in each group of treatment.

In **Paper I**, significances were tested by factorial analysis of variance (ANOVA) with genotype and diet as factors, to assess the effect of genetic depletion of *Soat2* independently of diet. Post-hoc comparisons were performed according to the Fisher´s least significant difference (LSD) test. The non-parametric Mann-Whitney U test was used to test differences in body weight.

In **Paper II**, the Mann-Whitney U test was used to test differences between the WT and *Soat2-/-* mice. Areas under the curve (AUC) were calculated by the trapezoid method and divided by time. The homeostasis model assessment (HOMA-IR) was calculated using the following equation: fasting blood glucose (mmol/L) × fasting plasma insulin (mU/L)/22.5[225]. The adipose tissue IR (Adipo-IR) was calculated as fasting plasma NEFA (mmol/L) × fasting plasma insulin (pmol/L)[226].

In **Paper III** and **IV**, significances were tested by Multi-Way ANOVA. Post-hoc comparisons were performed according to the LSD test.

In **Paper IV**, for RNA-sequencing data, likelihood ratio test (LRT) with reduced design formula was used for hypothesis tests. DE analyses were performed using the apeglm method implemented in DESeq2, and the significance was determined by p-value adjusted with the Benjamini-Hochberg method.

All statistical analyses were performed using Statistica versions 12.0 and 13.0 (StatSoft, Tulsa, OK), and RStudio version 1.1.383.

3.3 ETHICAL CONSIDERATIONS

Ethics are the code of conduct that distinguishes between right and wrong. Research is governed by these codes to which scientist must adhere. Hence, approval by ethical review board is needed to conduct any kind of research using biological experimental model. These ethical boards are guided by the well-recognized Declaration of Helsinki.

All animal and human studies were approved by the appropriate ethical boards as detailed below:

Mice: DNR: S9-10; DNR: S124-10; DNR: S130-12; DNR: S131-12

Human (Stockholm study): DNR: 2006/1204-31/1

Furthermore, the Stockholm study was registered at the EU clinical trial register (Eudract number: 2006-004839-30).

3.3.1 Ethical reflections from Papers I and II

Hepatic steatosis and insulin sensitivity are both complex diseases, involving several organs in collaboration with each other. Thus, studies in cell models cannot be used for these studies and experiments in humans are not allowed before convincing data has been obtained in animal models. We chose to use mice for these studies as they for long have been used in studies of cholesterol, TG and carbohydrate metabolism, which facilitate the interpretation of our results. We use eight mice per group (female & male) which is the lowest number of mice that we need to ensure statistically significant differences in insulin sensitivity tests. We also carefully plan and conduct the experiments to avoid repeating parts or the whole study. We strongly believed that the results of our experiments will not only increase the general understanding of the role of ACAT2 in the pathogenesis of hepatic steatosis and insulin sensitivity but also constitute the first pre-clinical evidence on the beneficial effect by inhibition of ACAT2, with minimal suffering for the animals.

3.3.1 Ethical reflections from Papers III and IV

Simvastatin and ezetimibe are commonly used to treat hyperlipidemic subjects. These two drugs are known to have some side effects. However serious side effects were not expected after four weeks of treatment and all subjects at high risk were excluded from the study. Also, all participants were free to drop out from the study without explanation.

Liver enzymes were analyzed to ensure safety before starting the study, and all adverse events were reported to decide if it jeopardized the participant health in order to stop his/her continuation in the study.

Liver biopsies were taken during the laparoscopic cholecystectomy, so no extra procedures were needed although there was still risk of bleeding which we tried to minimize by collaborating with senior surgeons and exclude any patients with higher bleeding risk.

Written consents were taken from all participants after fully explaining that the participation is voluntary and describing all the potential risks that could happen during participation.

There is no published data exploring the effects following treatment with ezetimibe either as mono- or in combination with simvastatin on hepatic cholesterol metabolism in human at molecular level.

We strongly believe that this study will not only provide new knowledge but also that the data obtained from this study will further support the recommendation of combination therapy in the treatment of dyslipidemia, with minimal risk for the subjects.

4 RESULTS AND DISCUSSION

4.1 REDUCED HEPATIC STEATOSIS IN *Soat2-/-* MICE

Alger *et al* has shown that *Soat2* depletion protects from dietary cholesterol-driven hepatic steatosis[65]; however, we hypothesized that *Soat2* depletion *per se* is protective from hepatic steatosis, independently of high dietary cholesterol, since most of the cholesterol in the intestine is coming from the bile[118]. In order to prove our hypothesis, we used three different diets (HFD, HCD and chow diet), all having low levels of cholesterol (<0.05%, w/w) (**Papers I** and **II**).

4.1.1 High-fat diet (HFD)

To investigate the role of *Soat2* depletion in hepatic steatosis, we fed female and male WT and *Soat2-/-* mice HFD with low-cholesterol content for eight weeks. Liver sections stained for the neutral lipids contained in LDs showed less intrahepatic lipid accumulation in *Soat2-/-* compared to WT mice. *Soat2-/-* female and male mice had significant lower hepatic TG (~ -30% and ~ -50% respectively), CE (~ -95% and ~ -95%, respectively) and FC (~ -15% and ~ -15%, respectively) levels compared to WT mice (Figure 2). We also found female mice to retain more hepatic CE compared to male mice (p<0.01) (**Papers I** and **II**).

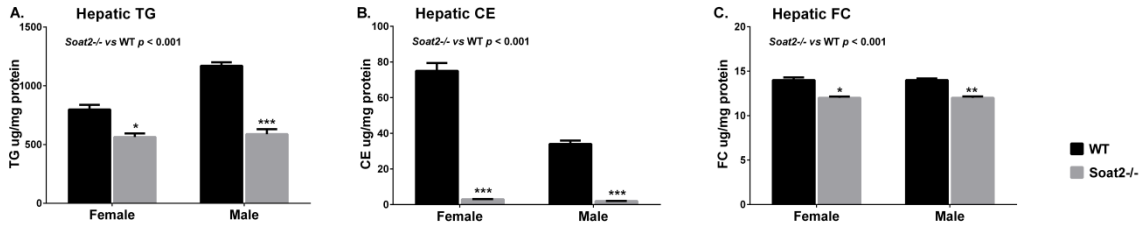

Figure 2. Reduced hepatic steatosis in *Soat2-/-* mice fed HFD. *Soat2-/-* and WT mice were fed HFD for eight weeks (n=8/group). A–C; quantification of hepatic TG, CE and FC levels showed reduced hepatic steatosis in *Soat2-/-* compared to WT mice. Data are expressed as mean ± SEM. Factorial ANOVA with genotype and diet as factors, followed by post-hoc comparison according to the LSD test; *p < 0.05, **p < 0.01 and ***p < 0.001.

4.1.2 High-carbohydrate (HCD) and chow diet

To further investigate the relevance of different dietary regimens on the pathophysiological role of *Soat2* depletion in hepatic steatosis, mice were also fed HCD or regular chow diet with low-cholesterol content.

As for HFD feeding, liver sections showed less intrahepatic lipid accumulation in *Soat2-/-* mice fed HCD compared to WT mice. *Soat2-/-* female and male mice had significant lower hepatic TG (~ -45% and ~ -75%, respectively) and CE (~ -85% and ~ -85%, respectively) levels compared to WT mice. As observed in HFD, female mice retain more hepatic CE compared to male mice (p<0.001). No significant differences in hepatic FC were present between *Soat2-/-* and WT mice; however, female mice had significantly higher hepatic FC (p<0.01) compared to male mice (Figure 3).

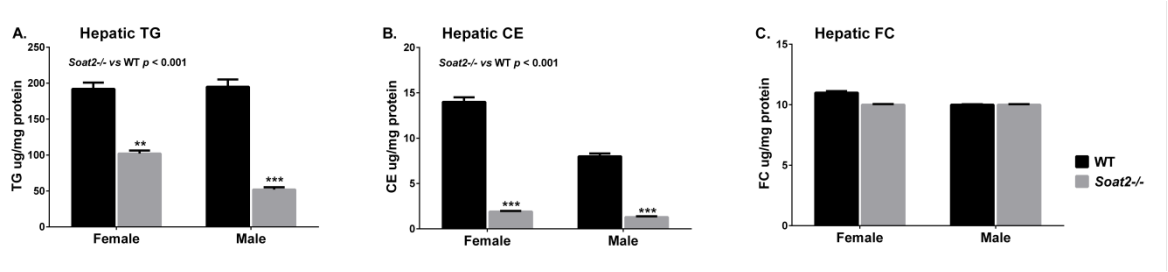

Figure 3. Reduced hepatic steatosis in *Soat2-/-* mice fed HCD. *Soat2-/-* and WT mice were fed HCD for two and half weeks (n=8/group). A–C; quantification of hepatic TG, CE and FC levels showed reduced hepatic steatosis in *Soat2-/-* compared to WT mice. Data are expressed as mean ± SEM. Factorial ANOVA with genotype and diet as factors, followed by post-hoc comparison according to the LSD test; *p < 0.05, **p < 0.01 and ***p < 0.001.

Female and male mice fed a regular chow diet have little intrahepatic lipids, yet we found *Soat2-/-* mice to have lower hepatic TG (~ -20% and ~ -40%, respectively) and CE (~ -55% and ~ -30%, respectively). In line with the findings in mice fed HFD and HCD, female mice accumulate more hepatic CE compared to male mice (p<0.01). Furthermore, higher hepatic FC (~ 40%) was observed in male *Soat2-/-* compared to WT mice (Figure 4) (**Papers I** and **II**).

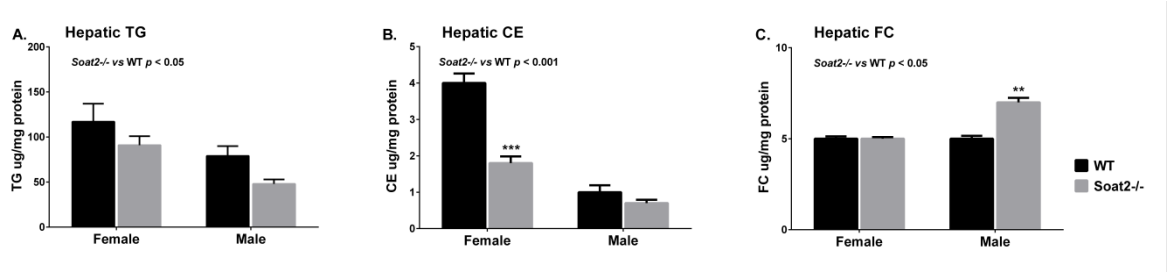

Figure 4. Reduced hepatic steatosis in *Soat2-/-* mice fed chow diet. *Soat2-/-* and WT mice were fed chow diet for eight weeks (n=8/group). A–C; quantification of hepatic TG, CE and FC levels showed reduced hepatic steatosis in *Soat2-/-* compared to WT mice. Data are expressed as mean ± SEM. Factorial ANOVA with genotype and diet as factors, followed by post-hoc comparison according to the LSD test; *p < 0.05, **p < 0.01 and ***p < 0.001.

No significant differences in weight-gain over the course of the study were observed between *Soat2-/-* and WT mice in all different dietary regimens.

4.1.3 Hepatic steatosis in all diets

Taken together, we found *Soat2* depletion to improve hepatic steatosis in all three different diets and independently of dietary cholesterol levels. The effect of *Soat2* depletion on hepatic CE is greater when the mice are fed HFD and HCD compared to chow diet due to a massive increase in hepatic CE; this suggests the protective role of *Soat2* depletion to be more pronounced when dietary insults are stronger. Although *Soat2* depletion significantly improves hepatic steatosis in both genders, female had higher levels of hepatic CE accompanied by less improvement in hepatic TG accumulation in all diets compared to male mice. These sex-related differences are in line with the higher ACAT2 enzymatic activity and *Soat2* gene expression in female mice and suggest sex-related differential response to *Soat2* depletion (see Figure 1).

4.2 IMPROVED INSULIN SENSITIVITY AND GLUCOSE TOLERANCE IN *Soat2-/-* MICE

As hepatic steatosis and IR go hand in hand (see section 1.1.3) and treatment of hepatic steatosis improves IR[8, 15], we next sought to investigate the effects by genetic depletion of *Soat2* on insulin sensitivity.

4.2.1 High-fat diet (HFD)

Oral glucose (OGTT) and insulin tolerance (ITT) tests showed that *Soat2-/-* mice were more insulin sensitive compared to WT mice, although the effects were stronger in male compared to female mice. Significant genotype-related effects were also present in serum glucose ($p<0.001$), insulin ($p< 0.05$) and HOMA-IR ($p<0.05$) with *Soat2-/-* having lower levels compared to WT mice.

Insulin suppresses the mobilization of NEFA from adipose tissue. *Soat2-/-* female mice had increased serum NEFA levels whereas *Soat2-/-* male mice showed no significant changes in NEFA levels and had lower adipo-IR compared to WT (Figure 5). This further indicates that *Soat2-/-* male mice had better insulin sensitivity compared to female mice (**Papers I** and **II**).

4.2.2 High-carbohydrate diet (HCD) and chow diet

OGTT and ITT in mice fed HCD showed improved ITT in *Soat2-/-* male mice compared to WT ($p<0.001$). Significant genotype-related effects were also present in serum glucose ($p<0.05$), insulin ($p< 0.01$) and HOMA-IR ($p<0.001$) with *Soat2-/-* having lower levels compared to WT (Figure 6).

In line with the findings in mice fed HFD, *Soat2-/-* female mice had increased serum NEFA levels whereas *Soat2-/-* male mice showed no significant changes. Moreover, *Soat2-/-* male mice had lower adipo-IR compared to WT and significant genotype-related effects were observed for adipo-IR ($p<0.001$).

In mice fed chow diet, *Soat2-/-* male mice showed improved ITT compared to WT with significant genotype-related effects in OGTT ($p<0.05$) and ITT ($p<0.01$). Moreover, no significant changes in glucose, insulin, HOMA-IR, NEFA and adipo-IR were observed between *Soat2-/-* and WT mice (**Papers I** and **II**).

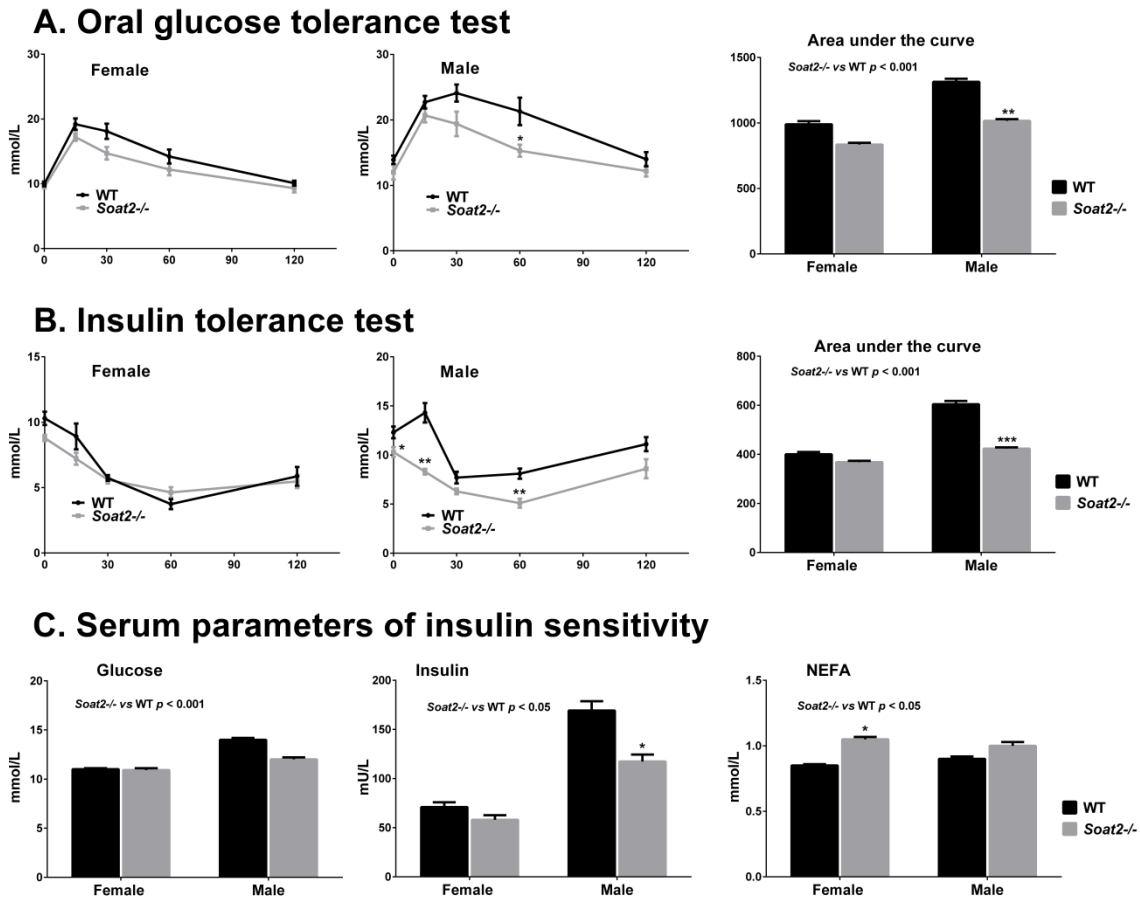

Figure 5. Improved glucose tolerance and insulin sensitivity in male *Soat2-/-* mice fed HFD. *Soat2-/-* and WT mice were fed HFD for eight weeks (n=8/group). A-B) After a four hour fast, mice were gavaged with glucose (OGTT) or injected (i.p.) with insulin (ITT) and glucose concentrations were monitored up to 120 minutes. Also, C) serum levels of glucose, insulin and NEFA were assessed as indicators of insulin sensitivity and HOMA-IR was calculated. Data are expressed as mean ± SEM. Factorial ANOVA with genotype and diet as factors, followed by post-hoc comparison according to the LSD test. For OGTT and ITT, Mann-Whitney U test was used to assess the differences between *Soat2-/-* and WT mice; *p < 0.05, **p < 0.01 and ***p < 0.001.

4.2.3 Insulin sensitivity and glucose tolerance in all diets

In concurrence with the prior findings, *Soat2* depletion clearly improved insulin sensitivity in male mice in all three different diets. In HFD and HCD, *Soat2-/-*male mice also showed signs of peripheral insulin sensitivity indicated by lower adipo-IR and absence of changes in NEFA levels compared to WT. On the other hand, the insulin sensitivity was not significantly improved in *Soat2-/-*female mice. However, *Soat2-/-*female mice showed ~ -50% reduction of fasting insulin while having similar fasting glucose levels as the WT, indicating an improved glucose tolerance when fed HCD.

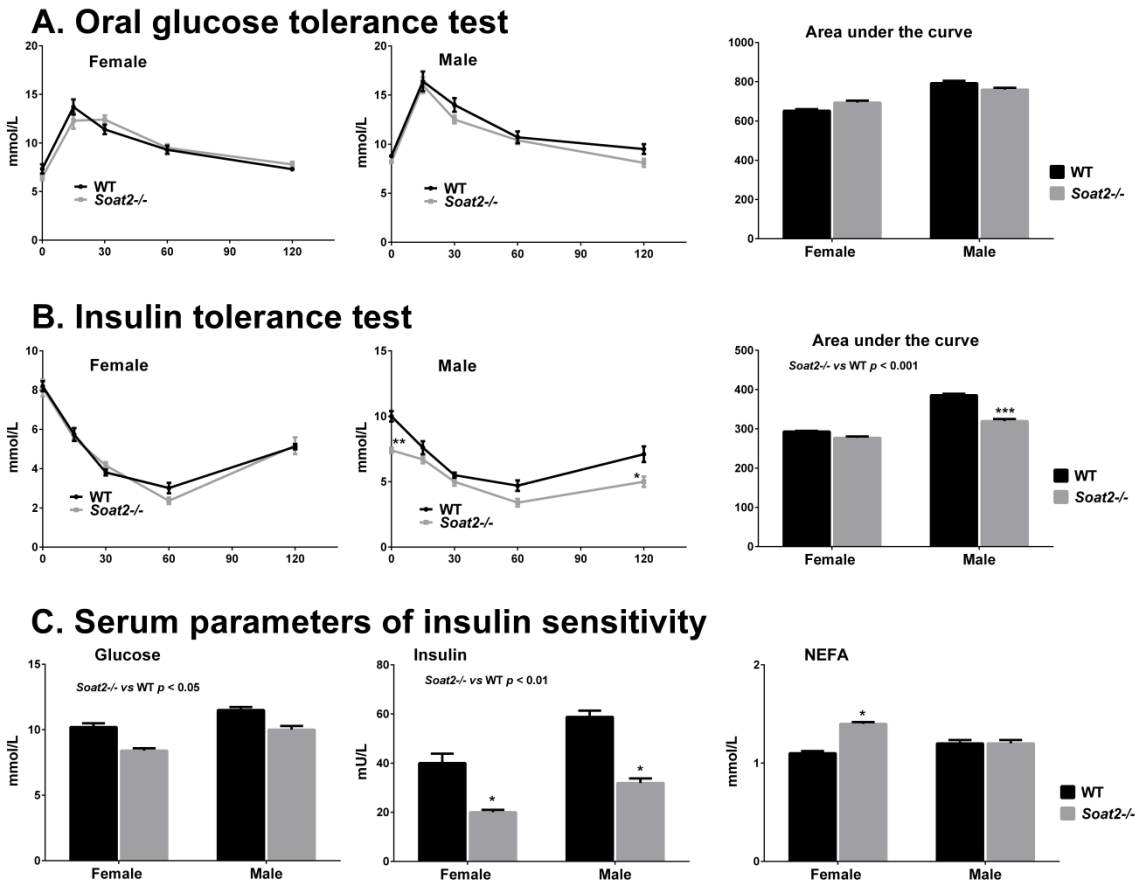

Figure 6. Improved glucose tolerance and insulin sensitivity in *Soat2-/-* mice fed HCD. *Soat2-/-* and WT mice were fed HFD for eight weeks (n=8/group). A-B) After a four hour fast, mice were gavaged with glucose (OGTT) or injected (i.p.) with insulin (ITT) and glucose concentrations were monitored up to 120 minutes. Also, C) serum levels of glucose, insulin and NEFA were assessed as indicators of insulin sensitivity and HOMA-IR was calculated. Data are expressed as mean ± SEM. Factorial ANOVA with genotype and diet as factors, followed by post-hoc comparison according to the LSD test. For OGTT and ITT, Mann-Whitney U test was used to assess the differences between *Soat2-/-* and WT mice; *p < 0.05, **p < 0.01 and ***p < 0.001.

4.3 POSSIBLE MECHANISMS OF REDUCED HEPATIC STEATOSIS IN *Soat2-/-* MICE.

4.3.1 Increased secretion of VLDL-TG

Alger *et al*[65] have reported an increased mobilization of TG from the liver, via increased secretion of VLDL-TG, in *Soat2-/-* mice fed high-cholesterol diet. In line with this, we found *Soat2-/-* mice fed HFD or HCD to have higher serum VLDL-TG concentrations compared to WT (**Papers I** and **II**). Moreover, male mice have higher serum VLDL-TG concentration compared to female mice when fed HFD, but no sex-related differences were present in mice fed HCD. However, no significant differences in serum VLDL-TG concentrations were observed in mice fed regular chow diet.

4.3.1.1 *Possible role of LD associate proteins*

As mentioned above (section 1.1.1.4.1), LD proteins play a crucial roles in VLDL secretion. Accordingly, we analyzed different LD proteins to uncover their potential role in protecting *Soat2-/-* mice from hepatic steatosis.

PLIN2 enhances TG storage and reduces VLDL-TG secretion[71]. Hepatic knockdown of *Plin2* resulted in reduced hepatic steatosis and increased VLDL-TG secretion in mice[72]. We observed significantly decreased *Plin2* mRNA levels in mice fed HFD compared to WT (Figure 7A); however, no major differences were observed on Plin2 protein levels. These changes at transcriptional level were more pronounced in male *Soat2-/-* mice and are in line with their levels of hepatic TG and serum VLDL-TG. In mice fed HCD, no changes in *Plin2* mRNA (Figure 7B) or protein levels were observed. The lack of effect on *Plin2* in *Soat2-/-* mice fed HCD may be due to insufficient stimulation of PPAR gamma, and thereby its target gene *Plin2*, by the low hepatic TG levels[227].

As mentioned above (section 1.1.1.3.2), CIDEC/ FSP27 is enriched at the contact points of LDs where it enhances lipid storage and prevents mobilization. Hepatic knockdown of *Fsp27* improves hepatic steatosis[44, 45] and glucose tolerance[47] in mice.

The hepatic *Cidec/ Fsp27* mRNA levels were significantly lower in male *Soat2-/-* mice fed HFD compared to WT mice (Figure 7A). The hepatic CIDEC/ FSP27 protein level was as much as 50% lower in *Soat2-/-* mice fed HFD. However, no significant changes in hepatic *Cidec/ Fsp27* mRNA levels were observed when fed HCD and regular chow diet. The reason for this discrepancy is probably due to the lower levels of hepatic lipid accumulation induced by these two diets compared to HFD. PPAR gamma, the main inducer of *Fsp27*[45], is expressed at very low levels in non-fatty livers[44]. The amount of hepatic TG in WT mice fed HFD were six to ten times higher than the levels found in WT mice fed HCD or chow diet and the *Fsp27* mRNA levels were 45 to 60 times higher in WT mice fed HFD compared to HCD or chow diet. Taken together, dietary fat enrichment is necessary to induce PPAR gamma and thereby its target gene *Fsp27*. Hence, depletion of *Soat2* seems to suppress the induction of *Fsp27* mRNA in mice fed HFD but does not affect the basal levels. Furthermore, in *Soat2-/-* mice fed HFD the ratio between FSP27 and PLIN2 proteins in LDs was dramatically changed and this may represent the molecular signal that promotes the mobilization of TG from LDs observed as consequence of *Soat2* depletion.

4.3.2 Reduced *de novo* lipogenesis (DNL)

Hepatic DNL plays an essential role in the pathogenesis of hepatic steatosis and NAFLD as well as IR (see section 1.1.1.1), and represents a promising target to treat these related conditions. To investigate the underlying molecular mechanisms account for the improved hepatic steatosis in *Soat2* deficient mice, key genes regulating DNL were analyzed. In female mice fed HFD or HCD, we found *Soat2* depletion to reduce the expression of genes involved in DNL (Figure 7) (**Paper I**). However, *Soat2-/-* male mice had lower levels of DNL regulating genes only when fed HCD (Figure 7B), which enhances DNL more strongly compared to HFD. Since inhibition of hepatic DNL was reported to enhance VLDL-TG secretion[15], probably this mechanism might contribute to the hypertriglyceridemia observed in *Soat2-/-* mice.

4.3.2.1 *Decreased hepatic GLUT2 membrane protein*

Since decreased GLUT2 levels has been associated to decreased DNL, improved hepatic steatosis and glucose tolerance in animal models[23] (see section1.1.1.1.1), we analyzed the GLUT2 protein levels. Indeed, *Soat2-/-* mice fed HFD or HCD had lower hepatic GLUT2 membrane protein levels compared to WT mice (~ -40 %) independently of sex (**Papers I** and **II**). As DNL uses glucose as a substrate for FAs synthesis, the decreased hepatic GLUT2 membrane protein levels would in turn lower DNL activity. Thus, *Soat2* depletion seems to lower the levels of DNL regulating genes and also the DNL precursor availability.

4.3.3 Increased fatty acid oxidation

Manipulation of hepatic FA oxidation affects hepatic steatosis in mice (see section 1.1.1.3). Since DNL and FA oxidation are closely regulated (see section 1.1.1.3.1), we assessed genes regulating hepatic FA oxidation. Analysis of some key genes suggested increased β-oxidation in *Soat2-/-* female mice which is in line with the results obtained by assessing DNL.

As FSP27 suppression increases the activity of mitochondrial β-oxidation and subsequently the TG turnover[45], it is plausible to speculate that *Soat2* depletion - at least with HFD - prevent hepatic steatosis through increased hepatic β-oxidation.

4.3.4 Decreased fatty acid uptake

CD36 mediates the hepatic uptake of FA and its hepatic expression has been reported to be correlated with hyperinsulinemia, hepatic steatosis, and IR in mice[32]. To further investigate the potential mechanisms responsible for reduced hepatic steatosis and improved glucose tolerance, hepatic mRNA levels of some key genes involved in hepatic FA metabolism were assessed. Genetic depletion of *Soat2* significantly lowers hepatic *Cd36* mRNA levels following feeding either HFD or HCD in male mice (Figure 7). However, *Soat2*-/- female mice lowers hepatic *Cd36* when fed HCD only (Figure 7B). Moreover, hepatic *Fabp4* mRNA level was significantly reduced in male mice fed HFD.

Taken together, modulation of FA uptake could be another mechanism by which *Soat2* deficiency improves hepatic steatosis and glucose tolerance, particularly in male mice.

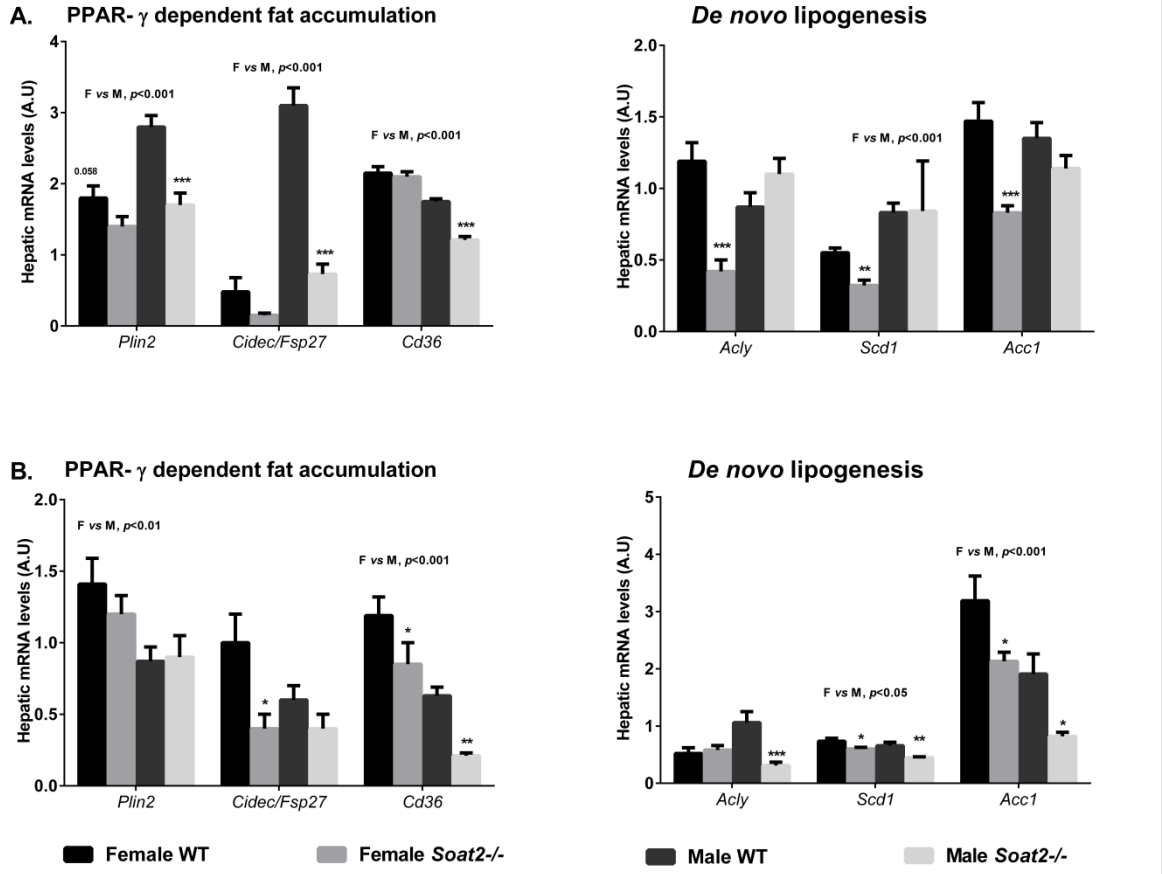

Figure 7. Possible transcriptional mechanisms of improved hepatic steatosis in *Soat2-/-* mice fed HFD and HCD. *Soat2-/-* and WT mice were fed HFD for eight weeks (n=8/group). A) Hepatic mRNA levels in mice fed HFD. B) Hepatic mRNA levels in mice fed HCD. Data are expressed as mean ± SEM. Factorial ANOVA with genotype and sex as factors, followed by post-hoc comparison according to the LSD test; *p < 0.05, **p < 0.01 and ***p < 0.001.

4.4 EFFECTS OF ADDING EZETIMIBE TO SIMVASTATIN ON REMNANT-CHOLESTEROL LEVELS

As mentioned above (see section 1.2.4), the circulating remnant-cholesterol consists of FC and CE carried in TG-rich lipoproteins (VLDL and chylomicrons). ACAT2 activity determines the content of CE secreted into TG-rich lipoproteins[109] whereas LCAT adds more CE to VLDL in plasma[228]. High-dose statin therapy decreases the activity of hepatic ACAT2[168] whereas ezetimibe inhibits the NPC1L1-mediated cholesterol availability for intestinal ACAT2 and consequently reduces CE in the core of chylomicrons, in mice[229]. Hence, we hypothesized that adding ezetimibe to simvastatin may enhance the lowering effect of simvastatin on remnant-cholesterol **(Paper III)**.

Indeed, we found additional reduction of remnant-cholesterol and remnant-CE (~ -15 %) when ezetimibe was added to simvastatin compared to simvastatin alone (Figure 8). Ezetimibe reduced CE in almost all remnant particles but not in all LDL subclasses. Thus, the additional reduction of CE in remnant particles by combined therapy supports the synergistic and complementary role of ezetimibe added to simvastatin. Moreover, ezetimibe added to

simvastatin showed significant additional reduction of apoB particles (~ -10 %) compared to simvastatin monotherapy.

Remnant-cholesterol can be deposited in the arterial wall by the interaction between apoB and PG[203]. Thus, the ability of lipid lowering drugs to reduce apoB particles could be as equally important as its ability to reduce LDL-C[202, 203].

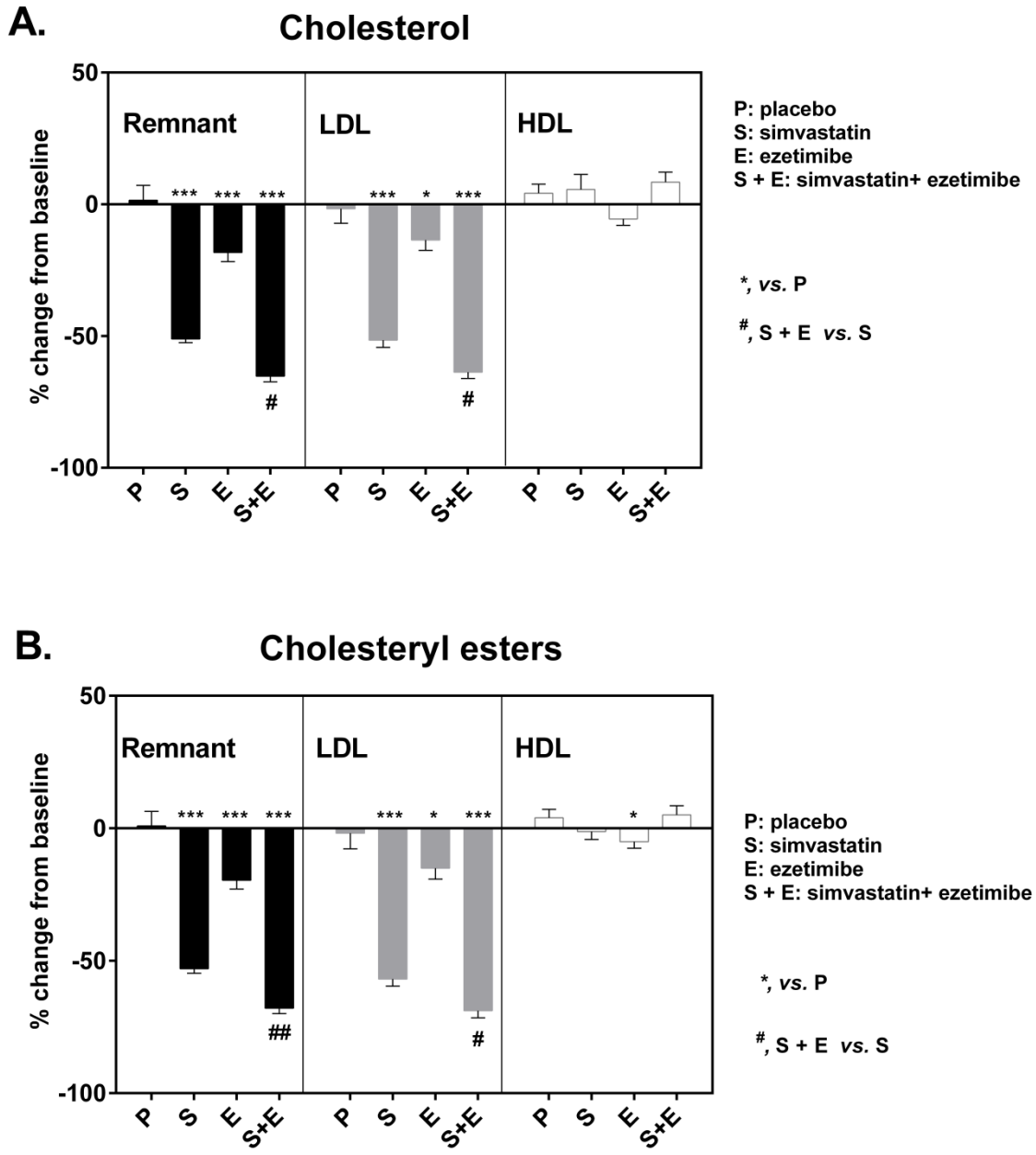

Figure 8. Effects of adding ezetimibe to simvastatin on remnant-cholesterol. A) Cholesterol and B) CE. Remnant particles i.e. non-high density lipoproteins (non-HDL) and non-low density lipoproteins (non-LDL). Data show the % change from baseline and are expressed as mean ± SEM. Fasted plasma lipoproteins were subjected to analysis by nuclear magnetic resonance. Multi-Way ANOVA followed by LSD-test. *vs. placebo, p <0.05; **vs. placebo, p <0.01; ***vs. placebo, p <0.001; #, simvastatin + ezetimibe vs. simvastatin, p <0.05 and ##, simvastatin + ezetimibe vs. simvastatin, p <0.01; (n=8-10/treatment group).

4.5 EFFECTS OF ADDING EZETIMIBE TO SIMVASTATIN ON PLASMA PROTEOGLYCAN (PG) BINDING

Both elevated remnant-cholesterol and LDL-C increase the risk for ASCVD (see section 1.2.4).Thus, the residual risk of ASCVD observed after optimal LDL-C lowering to the recommended level can partly be due to elevated remnant-cholesterol particles[202]. Our observation of significant additional reduction of remnant-cholesterol by combining ezetimibe to simvastatin, urged us to investigate additional athero-protective effects by assessing the binding of lipoproteins to human arterial PG (**Paper III**).

Both simvastatin and ezetimibe as monotherapy lowered the amount of plasma apoB-lipoprotein cholesterol bound to PG, although simvastatin was more effective. However, adding ezetimibe to simvastatin had no additional effects. We corrected the PG binding for cholesterol and apoB levels and found simvastatin still being able to reduce the binding capacity to arterial PG, but not ezetimibe. This data suggests that simvastatin remodel particles characteristics and not only numbers and cholesterol content.

4.6 EFFECTS OF ADDING EZETIMIBE TO SIMVASTATIN ON KEY GENES REGULATING HEPATIC CHOLESTEROL AND LIPOPROTEINS METABOLISM

In order to display the effects of adding ezetimibe to simvastatin on genes involved in cholesterol homeostasis, mRNA levels of some key genes were assessed in the liver.

As expected, SREBP2 target genes (*HMGCR*, *HMGCS1*, *LDLR*, *PCSK9* and *NPC1L1*) were significantly induced by simvastatin monotherapy (Figure 9). For most of the genes, adding ezetimibe to simvastatin has no effects, suggesting that cholesterol synthesis inhibition *per se* is sufficient to fully activate the SREBP2-system. Moreover, addition of ezetimibe to simvastatin therapy suppressed the induction of the hepatic mRNA levels of *MTTP* and *CETP*. These effects may partly explain how the combined therapy modifies the lipid composition of remnant particles compared to simvastatin monotherapy. Differently from our previous data with high-dose atorvastatin[168], no changes were seen in *SOAT2* mRNA levels. Due to lack of adequate biopsies we did not manage to assess the activity of hepatic ACAT2, which is a limitation of this study.

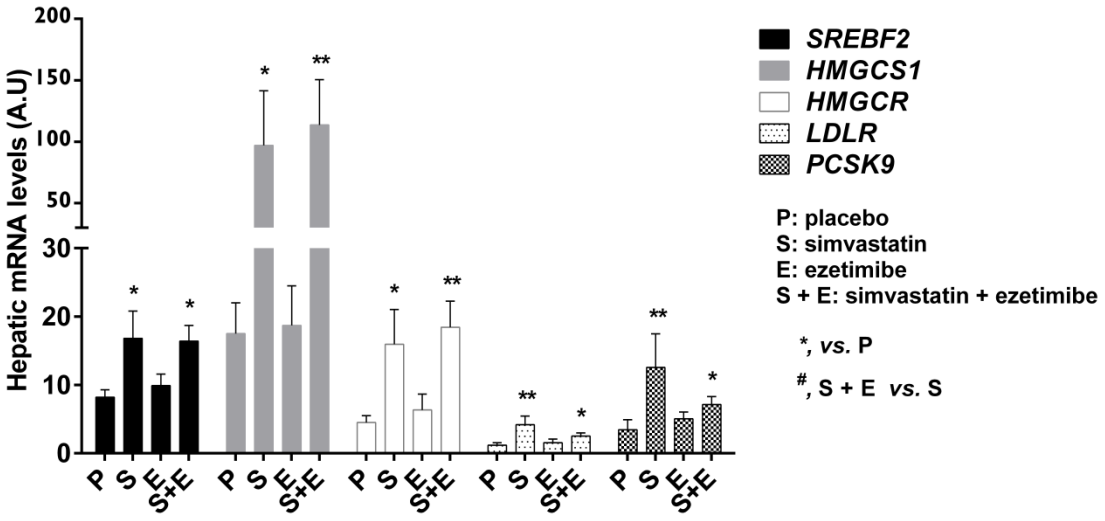

Figure 9. Effects of adding ezetimibe to simvastatin on genes involved in hepatic cholesterol and lipoproteins metabolism. A) Cholesterol, B. CE. Data are expressed as mean ± SEM. Multi-Way ANOVA followed by LSD-test. *vs. placebo, p <0.05; **vs. placebo, p <0.01; (n=5-9/treatment group).

4.7 MORE INSIGHTS ON THE ROLE OF HEPATIC NPC1L1

In the liver, Humans express NPC1L1 but its role for hepatic and biliary cholesterol homeostasis has not been elucidated, therefore we investigated hepatic NPC1L1 in **Paper III**.

We observed that hepatic cholesterol synthesis inhibition with simvastatin led to a compensatory decrease in biliary cholesterol due to the induction of hepatic NPC1L1. However, ezetimibe as monotherapy did not affect biliary cholesterol levels. This discrepancy is probably due to the low hepatic expression of NPC1L1 in gallstone disease patients[230], which may reduce the magnitude of ezetimibe effect.

Addition of ezetimibe to simvastatin created a new state where liver cannot compensate for the reduced cholesterol input from the intestine by *de novo* synthesizing cholesterol. In this new state, the biliary cholesterol concentration was similar to placebo indicating that hepatic NPC1L1 is probably a target for ezetimibe. If not, lower levels of biliary cholesterol should have been observed.

4.8 HEPATIC TRANSCRIPTIONAL RESPONSES TO EZETIMIBE AND SIMVASTATIN COMBINATION THERAPY

We have shown in **Paper III** that the addition of ezetimibe to simvastatin induced beneficial changes on apoB particles number and remnant-cholesterol content compared to simvastatin monotherapy. Therefore, we hypothesized that the difference in transcriptional profile in the combined therapy compared to simvastatin monotherapy to contribute to those beneficial changes (**Paper IV**).

In order to uncover the unexpected hepatic transcriptional changes, we evaluated global transcriptional profiles from liver biopsies taken from the subjects used in **Paper III**.

To validate the transcriptomic data, we first assessed SREBP2 target genes (see section 1.2.1) which are directly affected by simvastatin treatment. As expected, both the combination and simvastatin monotherapy induced those genes. Using a false discovery rate (FDR) of <0.05, we identified 260 reliable genes to be altered by different treatments. Out of these 260 treatment-responding genes; 39, 42 and 136 genes were enriched in the simvastatin, ezetimibe and combined treatment, respectively. Moreover, 95 genes were affected when ezetimibe added to simvastatin compared to simvastatin alone.

Mapping affected genes in different groups of treatment to REACTOME pathways displayed involvement of combined therapy in classical antibody-mediated complement activation. Both combined and simvastatin monotherapy showed higher expression levels of genes involved in hepatic cholesterol and lipid metabolism pathways. The gene ontology (GO) analysis also revealed the involvement of combination therapy in complement activation (classical pathway) and immune responses (q-value Bonferroni < 0.01).

Looking at individual genes, adding ezetimibe to simvastatin completely changed the pattern of expression of *MEP1B*, *CXCL13*, *ASCL1*, *GRPR* and *ANKRD22*. *TMC4* and *C3orf20* were downregulated with both combined and simvastatin monotherapy while *TFF3* and *SLC6A2* were significantly induced in response to the combination therapy, but not to the monotherapies (**Paper IV**).

Interestingly, an association between genetic variant of *MEP1B* and diabetic nephropathy was reported[231] whereas SNPs in *TMC4*[232] and *SLC6A2*[233] increase the risk of NAFLD. In mice, daily administration of recombinant TFF3 protein improves glucose tolerance[234]. Moreover, the upregulation of *SLC6A2*, which encodes a norepinephrine (NE) transporter protein, may explain the significant reduction of apoB particles observed in combined therapy compared to simvastatin alone (**Paper III**) since increased hepatic NE decrease apoB secretion[235].

In view of this data, adding ezetimibe to simvastatin seems to affect the predisposition to hepatic steatosis and NAFLD, and probably improve the glucose tolerance; however functional validation in bigger cohorts is needed.

5 CONCLUSIONS AND FUTURE CONSIDERATIONS

In the pre-clinical part of this thesis, we aimed to elucidate the changes at molecular levels that occur in mice after depletion of *Soat2* that leads to improved hepatic steatosis and insulin sensitivity. Using our knowledge from pre-clinical studies, we investigated the effects on remnant-cholesterol and hepatic gene expression following cholesterol absorption and/or cholesterol synthesis inhibition in humans.

In our pre-clinical studies, we propose different mechanisms to explain how depletion of *Soat2*, independently of the presence of high dietary cholesterol, protects from hepatic steatosis and improves insulin sensitivity and glucose tolerance.

We show that reduced hepatic CIDEC/FSP27 levels seem to be a relevant molecular change by which depletion of *Soat2* leads to improved hepatic steatosis and glucose tolerance in the sever fatty liver. Furthermore, we found reduced hepatic GLUT2 membrane protein levels which could be another molecular change by which *Soat2* depletion limits an important energy source of the liver, lowers DNL, and consequently decreases hepatic FA synthesis. The reduction of GLUT2 protein levels in liver membranes may also reduce hepatic glucose output which is an important contributor to fasting glucose levels and may well explain the improved glucose tolerance observed after *Soat2* depletion. Moreover, lower hepatic *Cd36* mRNA levels were observed particularly in *Soat2* male mice; thus modulation of FA uptake could also be considered as a protecting mechanism associated with *Soat2* depletion. *Soat2* depletion improves hepatic steatosis without negatively affecting skeletal muscle and adipose tissues.

In view of our hepatic mRNA data, we observed that most of the changes in *Soat2-/-* female mice seem to be driven by the SREBP1c pathway whereas the PPAR gamma pathway seems to regulate the changes in *Soat2-/-* male mice. Hence, *Soat2* depletion improves hepatic steatosis and glucose tolerance in both female and male mice, but the changes at molecular levels are sex-dependent, at least at the transcriptional level.

While I am writing, we are performing metabolic studies in *Soat2-/-* male mice to test our hypothesis that depletion of *Soat2* also leads to increased beta oxidation.

As mentioned above ACAT1 is located in at least one cell type in most tissues, while ACAT2 expression under physiological conditions is limited to enterocytes and hepatocytes. Similar to ACAT1, ACAT2 produces CE fated to storage in cytoplasmic LDs, but in contrast to ACAT1, ACAT2 also determines the amount of CE secreted in nascent apolipoprotein B-containing lipoproteins (i.e. chylomicrons and VLDL, respectively). Unfortunately, both *Soat1* and *Soat2* are expressed in HepG2, Huh7, and Huh7.5 cells and in primary human hepatocytes. Hence, none of these *in vitro* models resemble the human situation *in vivo*. Hence, they cannot be used to understand whether the molecular changes leading to increased mobilization of LDs occurring after genetic depletion of *Soat2* are relevant to the human condition. Therefore, we have now created hepatocytes-derived cells expressing only the ACAT2 protein in order to enable the translatability of our future

studies. The models will be used to study the molecular changes occurring after *Soat2* depletion and ACAT2 inhibition in a human hepatocyte-derived cell to further explore the link between cholesterol esterification, hepatic steatosis, and glucose tolerance.

The pre-clinical studies (**Papers I** and **II)**, showed that genetic depletion of *Soat2* significantly reduces hepatic steatosis and improves glucose tolerance via downregulation of hepatic GLUT2 membrane protein and dampened induction of FSP27 in the severe fatty liver. We proposed other sex-dependent mechanisms such as FA uptake and secretion via modulation of *Cd36* and *Plin2* mRNA levels, respectively in addition to DNL. Our study provides a link among hepatic cholesterol esterification, DNL as well as FA and glucose metabolism. Taken together, these data strongly support inhibition of ACAT2 activity as a promising target to treat CMD.

In our clinical study (**Paper III)**, we hypothesized that adding ezetimibe to simvastatin will greatly enhance the simvastatin lowering effect on circulating remnant-cholesterol. We further evaluated the effect of the combination therapy on binding to arterial PG. The mRNA levels of key genes involved in hepatic cholesterol homeostasis were assessed. We also characterized the role of hepatic NPC1L1 in light of the biliary cholesterol analysis and hepatic mRNA data.

We show that combining ezetimibe with simvastatin significantly reduce atherogenic apoB-lipoprotein content of remnant-cholesterol as well as the apoB particle numbers more efficiently than simvastatin alone. Since elevated remnant-cholesterol characterized patients with T2DM, this study gives mechanistic explanation of the improved survival among the subgroup of patients with T2DM treated with combined therapy compared with simvastatin alone in the IMPROVE-IT trial[149].

We propose combined therapy of simvastatin and ezetimibe as an optimal intervention for lipid disorders characterized by elevated remnant-cholesterol (i.e. T2DM) to reduce ASCVD in agreement with recent reports[148, 149] and meta-analysis[236].

Using a transcriptomic approach, we continued on investigating the potential beyond-lipid modification beneficial changes of adding ezetimibe to simvastatin therapy in the liver of the subjects recruited to our clinical trial (**Paper IV**). The results suggest that the combination therapy affects the regulation of the metabolically-mediated immune response which might further explain its beneficial effects over simvastatin monotherapy in reducing the risk of death from ASCVD. In view of the transcriptomic data, we speculate that adding ezetimibe to simvastatin affects individuals´ predisposition to NAFLD and might improve the glucose tolerance.

In the near future, we are planning to integrate the lipidomic results from **Paper III** with the DNA methylation data from liver biopsies of the same cohort which might reinforce our transcriptomic results and provide more insight whether the new genes identified in **Paper IV** are regulated at epigenetic level. Moreover, we will carry out single cell transcriptional

analysis to further characterize the hepatic transcriptional response to ezetimibe and/or simvastatin at different hepatic cellular subpopulations.

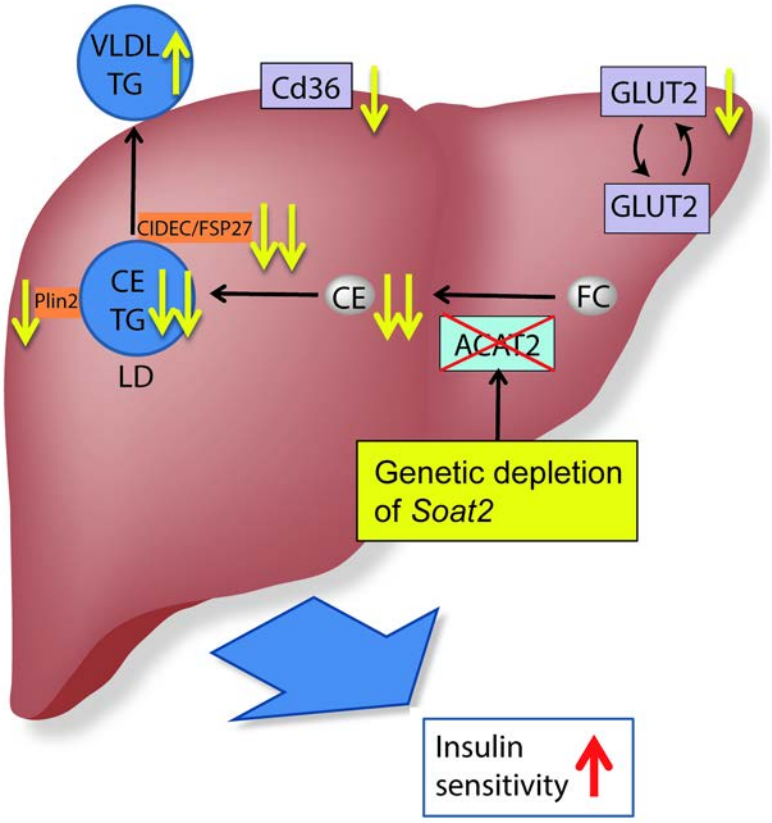

Figure 10. Graphical summary of effects of *Soat2* depletion on hepatic steatosis and insulin sensitivity in mice (**Papers I** and **II)**. Many thanks to *Tomas Jakobsson* for help in designing.

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