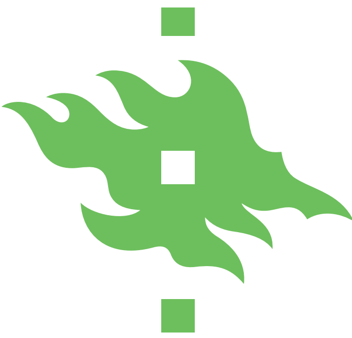

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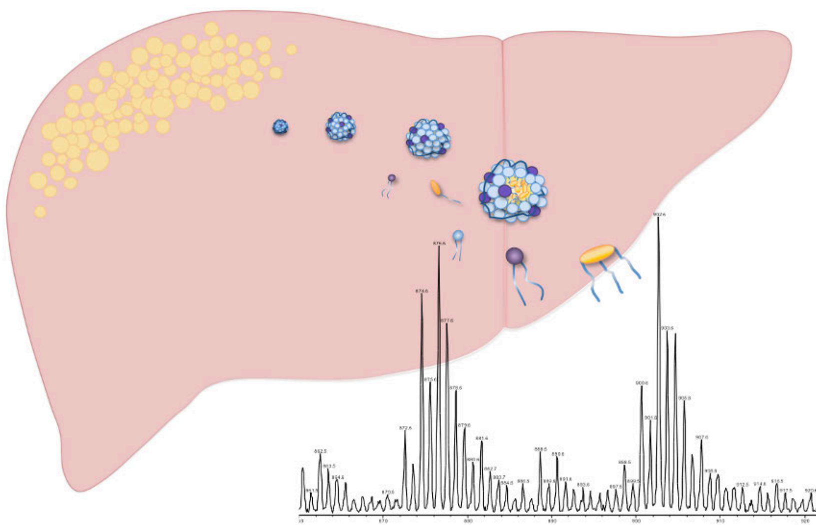
HANNA RUHANEN

HEPATIC LIPID METABOLISM IN CARDIOMETABOLIC DISEASES — PROTECTIVE AND ADVERSE EFFECTS OF GENETIC VARIANTS



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HEPATIC LIPID METABOLISM IN CARDIOMETABOLIC DISEASES – PROTECTIVE AND ADVERSE EFFECTS OF GENETIC VARIANTS

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ABSTRACT

Cardiometabolic diseases such as metabolic syndrome and non-alcoholic fatty liver disease (NAFLD) are risk factors for cardiovascular disease and type 2 diabetes. NAFLD can be seen as the hepatic manifestation of the metabolic syndrome and obesity increases the disease risk, but also a genetic component plays a role in the development of NAFLD. The I148M variant of PNPLA3 ($PNPLA3^{I148M}$) and E167K variant of TM6SF2 ($TM6SF2^{E167K}$) have been strongly associated with NAFLD. However, these variants cause a fatty liver without systemic metabolic complications, and $TM6SF2^{E167K}$ has even been shown to protect from myocardial infarction. New treatment possibilities for cardiovascular diseases have risen from studies of loss-of-function (LOF) variants of ANGPTL3. Subjects lacking ANGPTL3 have increased activity of lipoprotein lipase (LPL), low plasma levels of VLDL, LDL and HDL as well as increased insulin sensitivity.

In this thesis study we aimed to elucidate the function of PNPLA3 and TM6SF2 in lipid metabolism of human hepatocytes, and to clarify the mechanism underlying the association between the variants of these genes and increased hepatic lipid accumulation. We also investigated the function of ANGPTL3 in human hepatocytes and characterized the plasma lipoprotein lipidomes of subjects homozygous for ANGPTL3 LOF variants. In these studies, we utilized different lipidomics approaches as well as complementary methods such as microscopy and transcriptomics.

We found using labelled lipid precursors that overexpression of $PNPLA3^{I148M}$ in hepatocytes leads to a net accumulation of unlabelled triacylglycerols (TAGs) when compared to PNPLA3 wild type ($PNPLA3^{WT}$) overexpressing or control cells, but the level of newly synthesized TAGs did not change. Closer examination of the lipid species profiles and further experiments led us to the conclusion that PNPLA3 is a remodelling protein that transfers fatty acids from TAG to phosphatidylcholine (PC) and that $PNPLA3^{I148M}$ performs this function less efficiently, which may lead to increased hepatic TAG levels. The noticed lipid accumulation could also be related to a more extensive association of $PNPLA3^{I148M}$ to lipid droplets compared to $PNPLA3^{WT}$, which was also observed in our study.

We mimicked the effect of $TM6SF2^{E167K}$ by knocking down TM6SF2 in hepatocytes. TM6SF2 depletion increased the level of TAGs and cholesterol esters (CEs) and changed the membrane lipid composition of the cells by reducing the amount of polyunsaturated fatty acids (PUFAs) and increasing the levels of saturated and monounsaturated fatty acids in the lipids. The size of the lipoprotein-like particles secreted by the TM6SF2 deficient cells was reduced, as was β-oxidation of fatty acids. Both of these observations could explain the

increased lipid accumulation caused by TM6SF2 depletion. In addition, TM6SF2 knock-down increased lipid turnover and the amount of late endosomes/lysosomes in the cells.

Depletion of ANGPTL3 in hepatocytes lead to PUFA enrichment in major membrane phospholipids and CEs, and the production of PUFA-derived lipid mediators was also increased. In addition, the total level of CEs as well as their synthesis was reduced in ANGPTL3 depleted cells. An examination of the lipidome of lipoproteins derived from ANGPTL3 deficient or control subjects revealed that, in addition to reducing the total levels of all lipid classes, ANGPTL3 deficiency modifies the species composition of the core and surface lipids of lipoproteins, which likely reflects the increased activity of LPL.

These findings increase the knowledge on how genetic NAFDL caused by PNPLA3^{I148M} or TM6SF2^{E167K} variant develops and how ANGPTL3 depletion affects the liver and the secreted lipoproteins. This information provides tools for creating future prevention and treatment strategies for cardiometabolic diseases.

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LIST OF ORIGINAL PUBLICATIONS

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

I **Ruhanen H**, Perttilä J, Hölttä-Vuori M, Zhou Y, Yki-Järvinen H, Ikonen E, Käkelä R & Olkkonen VM (2014). PNPLA3 mediates hepatocyte triacylglycerol remodeling. *Journal of Lipid Research* 55: 739-746. DOI: 10.1194/jlr.M046607

II **Ruhanen H**, Haridas N, Eskelinen E-L, Eriksson O, Olkkonen VM & Käkelä R (2017). Depletion of TM6SF2 disturbs membrane lipid composition and dynamics in HuH7 hepatoma cells. *Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids* 1862: 676-685. DOI: 10.1016/j.bbalip.2017.04.004

III **Ruhanen H**, Haridas N, Minicocci I, Taskinen JH, Palmas F, di Costanzo A, D'Erasmo L, Metso J, Partanen J, Dalli J, Zhou Y, Arca M, Jauhiainen M, Käkelä R & Olkkonen VM (2020). ANGPTL3 deficiency alters the lipid profile and metabolism of cultured hepatocytes and human lipoproteins. *Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids* 1865:158679. DOI: 10.1016/j.bbalip.2020.158679

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AUTHOR'S CONTRIBUTION

I The author contributed in the design of the study, performed all the lipidomics experiments (including cell culture, lipid extraction, mass spectrometry, gas chromatography, data analysis and related protein normalization), and participated in writing and editing the article.

II The author contributed in the design of the study, created the knock-down and control cell lines, performed all the experiments and analyses except the ones including microscopy and thin layer chromatography, and participated in writing and editing the article.

III The author contributed in the design of the study, created the knock-down and control cell lines, did most of the cell culture work,prepared the RNA samples, performed all the ESI-MS/MS and gas chromatography related analyses, and participated in writing and editing the article

ABBREVIATIONS

ACAT	acyl-CoA:cholesterol acyltransferase
ANGPTL3	angiopoietin-like 3
Apo	apolipoprotein
CE	cholesterol ester
CETP	cholesteryl ester transfer protein
ChREBP	carbohydrate response element binding protein
DAG	diacylglycerol
EL	endothelial lipase
ER	endoplasmic reticulum
ESI-MS(/MS)	electrospray ionization (triple quadrupole) mass spectrometry
HDL	high density lipoprotein
HL	hepatic lipase
HMG	hydroxymethyl-glutaryl
IHH	immortalized human hepatocyte
KEGG	Kyoto encyclopedia of genes and genomes
LCAT	lecithin–cholesterol acyltransferase
LDL	low density lipoprotein
LOF	loss-of-function
LPL	lipoprotein lipase
MUFA	monounsaturated fatty acid
NAFLD	non-alcoholic fatty liver disease
NASH	non-alcoholic steatohepatitis
OCR	oxygen consumption rate
PA	phosphatidic acid
PLA	phospholipase A
PLS-DA	partial least squares discriminant analysis
PC	phosphatidylcholine
PCA	principal component analysis
PE	phosphatidylethanolamine
PI	phosphatidylinositol
PLTP	phospholipid transfer protein
PPAR	peroxisome proliferator-activated receptor
PS	phosphatidylserine
PUFA	polyunsaturated fatty acid
PNPLA3	patatin-like phospholipase domain containg 3
$PNPLA3^{WT}$	wild type PNPLA3
$PNPLA3^{I148M}$	rs738409 (I148M) variant of PNPLA3
SCD1	stearoyl-CoA desaturase-1, Δ9-desaturase
SD	standard deviation
SEM	standard error of the mean
SFA	saturated fatty acid

SIMCA	soft independent modelling of class analogy
SM	sphingomyelin
SPM	specialized pro-resolving mediator
SREBP	sterol regulatory element–binding protein
TAG	triacylglycerol
TM6SF2	transmembrane 6 superfamily member 2
$TM6SF2^{E167K}$	rs58542926 (E167K) variant of TM6SF2
VIP	variable importance in projection
VLDL	very low density lipoprotein

1 INTRODUCTION

A global rise in the prevalence of obesity has led to the increase in cardiometabolic diseases such as metabolic syndrome and related non-alcoholic fatty liver disease (NAFLD) (James et al. 2004, Diehl et al. 2019), which are risk factors for cardiovascular disease and type 2 diabetes (Wilson et al. 2005, Byrne and Targher 2015, Brouwers et al. 2020). The reasons behind the increasing rate of obesity are many and complex (Qasim et al. 2018), but undoubtedly the changes in lifestyle with less physical activity and diets high in saturated fat and sugar play an important role (James et al. 2004, Johns et al. 2015). Although in the last decades cardiovascular disease mortality has been falling in high-income countries, it has increased in low- and middle-income countries (Miranda et al. 2019), and the prevalence of NAFLD is still increasing worldwide (Younossi et al. 2016), as is the incidence of type 2 diabetes (Chatterjee et al. 2017). Cardiometabolic diseases are not merely a problem of the adult population since NAFLD can develop already in the childhood (Chalasani et al. 2018) and the pathological processes behind cardiovascular diseases can be set off during the first two decades of life (McGill et al. 2000). Hence different treatment and prevention options for these diseases are urgently needed, and the development of these new strategies requires deeper understanding of the mechanisms behind the conditions.

Genetic variants can both cause cardiometabolic diseases and prevent them from developing, thus providing possibilities for studying the mechanisms of disease development as well as prevention and treatment strategies. NAFLD is the most common cause of liver disease worldwide and it is considered as the hepatic manifestation of metabolic syndrome (Kotronen and Yki-Järvinen 2008). However, it is not a homogenous disease caused only by an unfavourable lifestyle, as genetics also plays a part in the disease risk. Variants of genes patatin-like phospholipase domain-containing 3 (PNPLA3) and transmembrane 6 superfamily member 2 (TM6SF2) cause a fatty liver disease that is in many ways different from the so called metabolic NAFLD that is associated with obesity (Romeo et al. 2008, Kozlitina et al. 2014). Genetic NAFLD caused by the I148M variant of PNPLA3 ($PNPLA3^{I148M}$) or the E167K variant of TM6SF2 ($TM6SF2^{E167K}$) is not associated with insulin resistance but is histologically more severe than the obesity-associated form of the disease (Kantartzis et al. 2009, Rotman et al. 2010, Liu, Y. L. et al. 2014, Zhou et al. 2015). Interestingly, $TM6SF2^{E167K}$ shows also cardioprotective effects as the lipids that would otherwise be secreted into circulation are retained in the liver (Holmen et al. 2014, Mahdessian et al. 2014). These variants of PNPLA3 and TM6SF2 were described in 2008 and 2014, respectively, and at the time of performing the studies described in publications I and II of this thesis, the functions of the wild type proteins as well as the mechanisms how their variants are causing NAFLD were not clear.

Loss-of-function (LOF) variants of angiopoietin-like 3 (ANGPTL3) are examples of genetic mutations that have evident cardiometabolic benefits protecting from the development of atherosclerotic cardiovascular disease (Dewey et al. 2017, Stitziel et al. 2017). ANGPTL3 is an inhibitor of lipoprotein lipase (LPL) (Shimizugawa et al. 2002), which hydrolyses

circulating lipoproteins (Merkel et al. 2002). Studies of subjects having no circulating ANGPTL3 suggest that complete ANGPTL3 deficiency induces a favourable plasma lipid profile characterized by distinct reduction of all plasma lipids with no evident complications (Minicocci et al. 2012, Stitziel et al. 2017), and two different therapeutic approaches of ANGPTL3 inhibition are already being tested in clinical drug trials. The first approach is a monoclonal antibody against the circulating protein and the other drug is an antisense oligonucleotide targeting hepatic ANGPTL3 (Dewey et al. 2017, Graham et al. 2017). Until now, only a limited amount of data has been published on the consequences of hepatic ANGPTL3 inhibition, and the effects of ANGPTL3 deficiency on the lipid profile of circulating lipoproteins have not been studied in detail.

The purpose of this thesis project was to clarify the functions of PNPLA3, TM6SF2 and ANGPTL3 in hepatic lipid metabolism (publications I, II and III, respectively). Also the mechanisms behind hepatic fat accumulation caused by the PNPLA3^{I148M} and TM6SF2^{E167K} variants were examined (publications I and II) and the detailed lipid profile of lipoproteins of ANGPTL3 deficient subjects was determined (publication III). Here, I first describe the physiological processes of hepatic lipid metabolism that were studied in publications I-III. Then I examine the aforementioned genetic variants in the context of cardiometabolic diseases and summarise the relevant literature on these variants. Finally, I present and discuss the findings of publications I-III.

2 REVIEW OF THE LITERATURE

2.1 Different roles of lipids

Lipids are classified based on their chemical structure, special focus being on their hydrophobic and hydrophilic components (Fahy et al. 2005). The chemistry of different lipids also defines their role in the cell; neutral lipids can be packed into lipid droplets and lipoproteins for storage and transport, respectively (Farese and Walther 2009, Tiwari and Siddiqi 2012), and amphipathic lipids are able to form membranes that allow the compartmentalization of a cell (van Meer et al. 2008) and provide precursors for signalling cascades (Wymann and Schneiter 2008).

2.1.1 Lipids as energy storage

All eukaryotic cells possess the ability to store lipids in specialized structures called lipid droplets (Ottaviani et al. 2011), which have a core of neutral lipids, namely cholesterol esters (CE) and triacylglycerols (TAG), surrounded by a phospholipid monolayer (Farese and Walther 2009). Vertebrates have also developed a dedicated cell type for storing lipids, the adipocytes (Ottaviani et al. 2011), which form the adipose tissue that is the most important long-term energy storage in mammals (Murphy and Vance 1999). During times of energy deprivation, lipid stores can be used for energy production; the hydrolysed acyl chains in β-oxidation, acetyl-CoA subsequently in ketogenesis, and the glycerol backbone in gluconeogenesis (Rui 2014). In addition to adipocytes, also other cell types, like hepatocytes and enterocytes, are able to store fat, but usually this storage is short-term and followed by secretion of neutral lipids in lipoproteins (Murphy and Vance 1999, Tiwari and Siddiqi 2012). If this balance is disturbed for example due to excess lipid accumulation in the adipose tissue i.e. obesity, lipids can start to accumulate in the liver leading to the development of a fatty liver (Vanni et al. 2010, Ipsen et al. 2018).

2.1.2 Lipids in membranes

Amphipathic lipids, which have both a hydrophilic and a hydrophobic element, form the basic structure of all membranes (van Meer et al. 2008). In lipid bilayers, such as the plasma membrane, endoplasmic reticulum (ER) and membranes of the Golgi and mitochondria, the hydrophilic head groups of the lipids point towards the aqueous environment while the hydrocarbon tails form a hydrophobic core of the membrane (van Meer and de Kroon 2011, Kimura et al. 2016). Phosphatidylcholine (PC) is the most abundant lipid in most mammalian membranes contributing roughly 50 % of the total phospholipids, the other major lipids contributing to the structure of the membrane being phosphatidylethanolamine (PE), phosphatidylserine (PS) and phosphatidylinositol (PI) (van Meer et al. 2008). There are also specialized areas called lipid rafts in cell membranes, which are enriched in other important membrane lipids sphingomyelin (SM), glycosphingolipids and cholesterol (Simons and

Ikonen 1997). The lipid rafts are thought to play a role in intracellular signalling (Simons and Ikonen 1997, Foster et al. 2003). In addition to having different microdomains, membrane bilayers are asymmetric; PC and SM are mainly distributed to the outer leaflet, and the main part of PE, PS and PI are found in the inner leaflet of the membrane facing the cytosol (Zachowski 1993). Cholesterol, however, can move readily between the bilayer leaflets compared to phospholipids and appears to be relatively evenly distributed between the inner and outer leaflet, although the distribution of cholesterol in the plasma membrane remains a matter of debate. (Bennett et al. 2009, Giang and Schick 2016, Steck and Lange 2018).

The structural properties and shape of phospholipids affect the packing and curvature of membranes. PC has a relatively large headgroup that together with the fatty acid tails gives it a cylinder-like shape, whereas PE has a smaller headgroup taking less space than its usually unsaturated acyl chains making PE a cone-shaped lipid that introduces negative curvature to membranes (Marsh 2007, van Meer et al. 2008, Somerharju et al. 2009). The packing of a membrane is affected by both the headgroups of lipids (Somerharju et al. 2009) and their fatty acid tails, unsaturated fatty acids making the membrane more fluid (Stubbs and Smith 1984, Small 1984). The properties of membranes define the environment for proteins, and lipid-protein interactions also affect the stability and function of integral and transmembrane proteins (Marsh 2007).

2.1.3 Lipids in cellular signalling

Membranes are important sites of cellular signalling, and especially lipid raft areas are enriched in proteins involved in signalling processes (Simons and Ikonen 1997, Foster et al. 2003). Although individual lipid rafts are small, 10–200 nm in size, they can compose a relatively large proportion of the plasma membrane (Hao et al. 2001, Pike 2003, Pike 2006). Rafts are not identical in their protein composition and they can gather together functional assemblies of proteins dedicated for specific tasks like cellular signalling (Pike 2003, Foster et al. 2003). Lipid rafts are thought to participate in controlling signal transduction in many ways. For example, rafts containing different signalling proteins can fuse thus activating signalling pathways, or quite the contrary, rafts can inactivate signalling by creating spatial segregation of interacting components (Pike 2003).

Different types of phospholipases hydrolyse amphipathic lipids yielding both hydrophobic and hydrophilic molecules that can transmit a signal within the membrane and through the cytosol, respectively (Dennis et al. 1991, van Meer et al. 2008). Phospholipase A_1 (PLA1) mediated hydrolysis releases a fatty acid from the first carbon or *sn*-1 position of the glycerol backbone of a phospholipid producing for example lysoPA, which is an active mediator of lipid signalling (Aoki et al. 2007, Meyer zu Heringdorf and Jakobs 2007). Similarly, different phospholipase A_2 (PLA2) isoforms free a fatty acid from the *sn*-2 position of a phospholipid yielding also a corresponding lysolipid (Burke and Dennis 2009). The fatty acid in the *sn*-2 position is usually a polyunsaturated fatty acid (PUFA) (MacDonald and Sprecher 1991), which can be used for the production of bioactive lipid mediators (Buckley et al. 2014, Dennis

and Norris 2015). These eicosanoids and docosanoids play a comprehensive role for example in the initiation and resolution of acute inflammation (Buckley et al. 2014, Dennis and Norris 2015).

Phospholipase C cleaves the bond between the glycerol backbone and the phosphate group of a phospholipid (Dennis et al. 1991). The function of phospholipase C on phosphorylated PI derivatives releases inositol phosphates and diacylglycerols (DAGs), which are both intracellular second messengers with a wide range of downstream effects (Berridge 2016). Sphingomyelinases remove the phosphocholine headgroup of SM releasing ceramide, which can again be metabolised into sphingosine, sphingosine-1-phosphate and ceramide-1-phosphate, all of which possess signalling capacity (Futerman and Hannun 2004). Phospholipase D acts mainly on PC releasing choline and phosphatidic acid (PA), but it also hydrolyses other phospholipids (Dennis et al. 1991, Wang et al. 2006). PA is targeting for example proteins involved in vesicular trafficking, G protein regulation and phosphorylation/dephosphorylation of proteins and lipids (Wang et al. 2006). Disruption or imbalance of lipid signalling pathways can lead to many adverse effects, such as development of chronic inflammation, metabolic syndrome, atherosclerosis and cancer (Wymann and Schneiter 2008).

2.2 Hepatic lipid metabolism

The liver is a motor of human metabolism. It orchestrates important metabolic functions such as lipid synthesis and oxidation, which are also coupled to glucose metabolism (Rui 2014). The liver is able to shunt excess energy derived from carbohydrate and protein into *de novo* lipogenesis in the form of acetyl-CoA (Acheson et al. 1988, Charidemou et al. 2019), and is a crucial player in lipoprotein metabolism as it both takes up and secretes lipoproteins (Jones et al. 1984, Tiwari and Siddiqi 2012).

2.2.1 Lipid synthesis

In addition to the adipose tissue and intestine, the liver is a key site of lipid synthesis in humans (Rui 2014). The ER is the organelle where a majority of the reactions of lipid synthesis occur, but also Golgi, mitochondria and peroxisomes play a part in the process (Fagone and Jackowski 2009). Lipid synthesis is under both hormonal and transcriptional regulation (Wang and Viscarra et al. 2015).

2.2.1.1 Fatty acid synthesis

In mammals, fatty acid synthesis takes place mainly in the liver, adipose tissue and lactating mammary gland (Pearce 1983). Acetyl-CoA, the starting substrate in the process, can be derived originally from either carbohydrate or protein sources (Acheson et al. 1988,

Charidemou et al. 2019). An enzyme complex named fatty acid synthase is responsible for a series of reactions in which acetyl-CoA, malonyl-CoA and NAPDH are utilized to yield mainly palmitate (16:0) (Jensen-Urstad and Semenkovich 2012). Palmitate can be further elongated by elongases that add two-carbon units from acetyl-CoA into the carboxyl end of the fatty acid, and desaturated by the function of Δ9-desaturase (stearoyl-CoA desaturase-1, SCD1) (**Figure 1**) (Guillou et al. 2010). It should be noted that essential fatty acids linoleic acid (18:2n-6) and α-linolenic acid (18:3n-3) must be obtained from the diet since they cannot be synthesized *de novo*. These essential fatty acids can, however, be further elongated and desaturated by Δ6- and Δ5-desaturases (fatty acid desaturase, FADS1 and 2, respectively) to yield long-chain and very long-chain n-6 and n-3 series polyunsaturated fatty acids (**Figure 1**) (Guillou et al. 2010).

De novo fatty acid synthesis is under hormonal control of insulin and glucagon, and the activity of enzymes involved in the fatty acid synthesis is adjusted mainly by transcriptional control of glycolytic and lipogenic genes (Horton et al. 2002, Rui 2014, Wang and Viscarra et al. 2015). Glucose stimulates carbohydrate response element binding protein (ChREBP), which activates lipogenic genes such as fatty acid synthase and stearoyl-CoA desaturase-1 (Iizuka 2017). Insulin induces and glucagon downregulates sterol regulatory element–binding proteins (SREBPs) that are also referred to as master regulators of lipid metabolism (Eberle et al. 2004, Rui 2014). In addition to activating fatty acid synthesis, SREBPs also increase the expression of the key genes of cholesterol synthesis (Eberle et al. 2004).

Figure 1. Simplified overview of fatty acid synthesis and *de novo* glycerophospholipid synthesis (Shindou and Shimizu 2009, Guillou et al. 2010). GPAT=glycerol-3-phosphate acyltransferase, AGPAT=acylglycerol-phosphate acyltransferase, LPAAT=lysophosphatidic acid acyltransferase, PAP=phosphatidic acid phosphatase, DGAT=diacylglycerol acyltransferase, DAG=diacylglycerol, TAG=triacylglycerol, PC=phosphatidylcholine, PE=phosphatidylethanolamine, PS=phosphatidylserine, PI=phosphatidylinositol, PG=phosphatidylglycerol.

2.2.1.2 TAG synthesis

The main sites of *de novo* TAG synthesis are the liver and adipose tissue where TAG is produced via the glycerol-3-phosphate (i.e. Kennedy) pathway in the ER of the cells (Lehner and Kuksis 1996, Ameer et al. 2014). In the liver, the glycerol-3-phosphate needed for this pathway is derived from plasma glycerol by the action of glycerol kinase or by the reduction of a glycolytic intermediate dihydroxyacetone phosphate, and from glyceroneogenesis in which glycerol is produced *de novo* from pyruvate (Kalhan et al. 2001). Fatty acids used in TAG synthesis are either synthesized *de novo* or derived from dietary lipids or endogenous adipose tissue (Lehner and Kuksis 1996). Fatty acids are incorporated into to the glycerol-3-phosphate backbone in a stepwise manner (**Figure 1**). First *sn*-1-glycerol-3-phosphate acyltransferase catalyses the formation of lysophosphatidic acid, which in turn is acylated into PA by *sn*-1-acylglycerol-3-phosphate acyltransferase (Lehner and Kuksis 1996, Coleman and Mashek 2011). Next PA phosphatase hydrolyses PA to form DAG, which is finally esterified into TAG by DAG acyltransferase (Lehner and Kuksis 1996, Kalhan et al. 2001, Coleman and Mashek 2011). TAG can also be produced from monoacylglycerol by the function of monoacylglycerol acyltransferase and DAG acyltransferase (Quiroga and Lehner 2012) and it has been suggested that glycerol could be directly acylated into monoacylglcerol in mammalian tissues through a direct acylation pathway (Lee et al. 2001).

2.2.1.3 Phospholipid synthesis

The first steps of *de novo* synthesis of glycerophospholipids are the same as described above for TAG synthesis and depending on the phospholipid class the pathways diverge once PA or DAG has been synthesized (**Figure 1**). A majority of the reactions of phospholipid synthesis take place in the ER, but also the Golgi, mitochondria and peroxisomes have their roles in the process (Fagone and Jackowski 2009). PI, phosphatidylglycerol, and cardiolipin are synthesized from cytidine diphospho-DAG, which is derived from PA (Shindou and Shimizu 2009, Blunsom and Cockcroft 2020), while the two most abundant phospholipids of mammalian cells, PC and PE, are synthesized from DAG (Smith et al. 1957, Bleijerveld et al. 2007). Ether PC and PE, which are defined by an ether bond at the *sn*-1 position of the glycerol backbone, are derived from an acylated form of dihydroxyacetone phosphate through the function of peroxisomal enzymes (van den Bosch and de Vet 1997). In mammalian cells, phosphatidylserine (PS) is synthesized solely though exchanging the head-group of an existing phospholipid for L-serine (Kuge and Nishijima 1997), and inversely, PE can also be derived from decarboxylation of PS in the mitochondrial membrane (Vance 1990, Bleijerveld et al. 2007).

SM is a sphingolipid analogue of PC since it has a phosphorylcholine headgroup attached to the sphingoid base component of a ceramide. SM is formed when SM synthase transfers a phosphorylcholine headgroup from PC to ceramide yielding SM and DAG (Gault et al. 2010). SM synthases are present in the Golgi and plasma membrane (Gault et al. 2010).

2.2.1.4 Cholesterol and cholesterol ester synthesis

The liver and small intestine are the main sites of cholesterol synthesis in humans (Dietschy and Wilson 1970). The synthesis occurs through a so-called mevalonate or isoprenoid pathway and requires a complex series of enzymatic reactions (Bloch 1965, Goldstein and Brown 1990, Russell 1992). The process starts by condensation of two acetyl-CoAs into acetoacetyl-CoA, after which hydroxymethyl-glutaryl (HMG)-CoA is synthesized from the formed acetoacetyl-CoA and acetyl-CoA (Russell 1992, Cerqueira et al. 2016). The subsequent step yielding mevalonate by the action of HMG-CoA reductase is highly regulated and is considered as the rate-limiting step of the pathway (Goldstein and Brown 1990, Russell 1992, Cerqueira et al. 2016). However, balancing the endogenous cholesterol synthesis and exogenous cholesterol uptake also requires the regulation of other enzymes of the pathway, especially HMG-CoA synthase and squalene monooxygenase, as well as the control of low density lipoprotein (LDL) receptors (Goldstein and Brown 1990, Russell 1992, Gill et al. 2011, Cerqueira et al. 2016). The final product of the mevalonate pathway, cholest-5-en-3β-ol or cholesterol, is a sterol having a tetracyclic structure and one side chain (Cerqueira et al. 2016).

CEs are synthesized from cholesterol and CoA esters of fatty acids in the ER of hepatocytes and most other mammalian cell types by two isoforms of acyl-CoA:cholesterol acyltransferase (ACAT) (Erickson and Cooper 1980, Anderson et al. 1998, Oelkers et al. 1998, Korber et al. 2017). In human liver *in vivo*, ACAT2 is the major isoform (Parini et al. 2004), and it is found only in the liver and intestine while ACAT1 is more widely expressed (Anderson et al. 1998, Oelkers et al. 1998). In plasma high density lipoproteins (HDL) and LDL, CEs are synthesized by the function of lecithin–cholesterol acyltransferase (LCAT), which transfers a fatty acid to cholesterol from the *sn*-2 position of PC, thus also yielding lysoPC (Glomset 1962, Chen and Albers 1982).

2.2.1.5 Lipid mediator synthesis

The synthesis of bioactive lipid mediators begins when a lipase, like cytosolic PLA2, releases PUFAs from glycerolipids (Murakami et al. 2011, Dichlberger et al. 2014, Batchu et al. 2016). Cyclooxygenases, lipoxygenases and cytochrome P450 enzymes then act upon these PUFA substrates, such as 20:4n-6, 22:4n-6, 20:5n-3, 22:5n-3 and 22:6n-3, to produce eicosanoids and docosanoids like prostaglandins, thromboxanes, leukotrienes, lipoxins, resolvins, protectins and maresins (**Figure 2**) (Buckley et al. 2014, Dennis and Norris 2015). The n-6 series-derived lipid mediators are synthesized as a response to infection or tissue injury so most of them are pro-inflammatory and are needed for the onset of a normal inflammatory response (Ricciotti and FitzGerald 2011, Dennis and Norris 2015). In addition, prostaglandins are produced during the initiation of the resolution phase of inflammation (Levy et al. 2001), and lipoxins derived also from 20:4n-6 are classified as pro-resolving mediators (Pirault and Bäck 2018). The n-3 series derived specialized pro-resolving mediators resolvins, protectins

and maresins are synthesized after a lipid mediator class-switching stimulus of prostaglandins (**Figure 2**) (Levy et al. 2001, Buckley et al. 2014, Serhan et al. 2014).

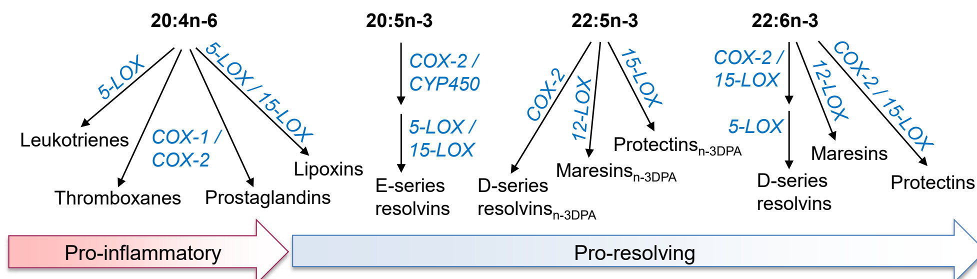

Figure 2. Simplified overview of the synthesis of 20:4n-6, 20:5n-3, 22:5n-3 and 22:6n-3 derived pro-inflammatory and pro-resolving lipid mediators (Dalli et al. 2013, Serhan et al. 2014, Lopez-Vicario et al. 2016, Pistorius et al. 2018, Recchiuti et al. 2019). LOX=lipoxygenase, COX=cyclooxygenase, CYP450=cytochrome P450, DPA=Docosapentaenoic acid.

2.2.2 Lipid remodelling

Lipid remodelling is a process in which a fatty acid esterified to the backbone of the lipid is removed and replaced by another fatty acid without otherwise changing the structure of the lipid. The concept of glycerolipid remodelling was first introduced by Lands in 1958 (Lands 1958). Today, three different enzyme systems are known to be responsible for remodelling of phospholipids: acyl-CoA:lysophospholipid acyltransferases, and CoA-dependent and CoA-independent transacylation systems (Yamashita et al. 2013). Acyl-CoA:lysophospholipid acyltransferases function in a deacylation-reacylation reaction in which PLA1 or PLA2 first cleaves the fatty acid at the *sn*-1 or *sn*-2 position respectively, thus yielding a lysophospholipid which is then re-esterified with a different fatty acid by an acyl-CoA:lysophospholipid acyltransferase (MacDonald and Sprecher 1991, Yamashita et al. 2013). There are several different acyl-CoA:lysophospholipid acyltransferases involved in the remodelling pathways, some of them specific for a certain phospholipid class and others functioning more broadly (Shindou and Shimizu 2009, Yamashita et al. 2013). The function of this type of a remodeling enzyme can also be fatty acid selective; for example lysophosphatidylcholine acyltransferase 3 enriches arachidonate in the *sn*-2 position of membrane phospholipids (Hashidate-Yoshida et al. 2015).

Also CoA-dependent transacylation systems have been shown to possess fatty acid specificity in mammalian liver, transferring distinctively fatty acids 20:4n-6, 18:2n-6 and 18:0 (Sugiura et al. 1988, Sugiura et al. 1995). Yamashita et al. (2013) propose a mechanism for CoA-dependent transacylation in which an acyl chain is removed from a donor phospholipid by the reverse reaction of an acyl-CoA:lysophospholipid acyltransferase and attached to CoA followed by reacylation to an acceptor lysophospholipid by a forward reaction of the same family of enzymes. The mechanism of function of CoA-independent transacylation is not well established, but it is possibly mediated by PLA2 (Yamashita et al. 2017). CoA-independent

transacylases have been shown to catalyse the transfer of 20:4n-6 and 22:6n-3 to *sn*-2 position of phospholipids (Kramer and Deykin 1983, Sugiura et al. 1985), however this activity is low in mammalian liver (Sugiura et al. 1988).

Fatty acids are also recycled in TAG (Lankester et al. 1998, Reshef et al. 2003, Quiroga and Lehner 2012). In mammals, fatty acids are circulated in a TAG/fatty acid cycle, in which fatty acids released from the adipose tissue are re-esterified into TAG in the tissue of origin or in the liver (Reshef et al. 2003). Approximately 60% of the fatty acids released from the adipose tissue are shunted into this cycle (Reshef et al. 2003). TAGs can also be hydrolysed for re-esterification in hepatocytes by several lipases, the function of which is however not fully established (Quiroga and Lehner 2012). One of these enzymes is PNPLA3, whose role in TAG remodelling is examined in publication I of this thesis.

2.2.3 Lipoprotein metabolism

Lipoproteins transport dietary and endogenously synthesized lipids in the circulation. They carry their cargo in the hydrophobic core of the particle, which is surrounded by a monolayer of PC, lysoPC, SM and cholesterol and attached apolipoproteins (Francis 2016, McLeod and Yao 2016). Lipoproteins are modified in the circulation and their lipids hydrolysed in order to deliver fatty acids to tissues (Wang et al. 2013). Lipoprotein remnants are taken up by the liver, which again secretes new lipoproteins into the circulation (Jones et al. 1984, Tiwari and Siddiqi 2012).

2.2.3.1 Chylomicrons

Chylomicrons are synthesized in enterocytes and secreted from the intestine into the circulation via the lymphatic system (Hussain 2014). Most of dietary lipid is TAG so the lipids entering the enterocytes from the intestinal lumen are mainly fatty acids and monoacylglycerols yielded by the action of pancreatic lipase (Iqbal and Hussain 2009). They are reassembled in the ER, and thus also the secreted chylomicron particles contain mainly TAG (Iqbal and Hussain 2009). Also some cholesterol is packed into the chylomicrons as CEs by the function of ACAT2 (Buhman et al. 2000, Iqbal and Hussain 2009). Chylomicrons contain one apolipoprotein B48 (ApoB-48), which is an intestinal variant of apolipoprotein B100 (Apo-B100) found in very low density lipoproteins (VLDLs) and LDLs (Chen et al. 1987). ApoB-48 is needed for the assembly and secretion of the chylomicron particles together with ApoA-IV and microsomal triglyceride transfer protein (Iqbal and Hussain 2009, Hussain 2014).

In the circulation TAG carried in chylomicrons is hydrolysed by LPL attached to heparan sulphate proteoglycans of endothelial cells of vessel (Olivecrona 2016). The formed chylomicron remnants can be further hydrolysed by hepatic lipase (HL) (Santamarina-Fojo et al. 2004) or delivered directly to the liver for uptake (Jones et al. 1984).

2.2.3.2 VLDL and LDL

VLDLs are secreted by the liver in a similar process as described for chylomicrons. VLDL secretion requires a supply of TAG in the ER, where the particle is assembled (Shelness and Sellers 2001, McLeod and Yao 2016), and a favourable membrane composition, 20:4n-6-containing PCs being important membrane components (Rong et al. 2015). Also ApoB-100 and microsomal triglyceride transfer protein are required for the formation of a VLDL particle, and ACAT2 is needed for secretion of cholesterol as CE in VLDL (Buhman et al. 2000). The VLDL particle is smaller than a chylomicron, but the compositions of these lipoproteins show similarity, as TAG is the most abundant lipid also in VLDL (McLeod and Yao 2016). Hence, chylomicron and VLDL particles are often referred to as triglyceride-rich lipoproteins.

Nascent VLDL particles are transported from the ER and through the Golgi where their apolipoproteins are modified, after which the mature VLDL is secreted to the plasma membrane by a vesicular system (Tiwari and Siddiqi 2012, Hossain et al. 2014). VLDL particles are hydrolysed in the circulation in the same way as chylomicrons by the function of LPL, yielding VLDL remnants (Khetarpal and Rader 2015, Olivecrona 2016). LDL is formed when these remnants are further processed by LPL, first into intermediate density lipoproteins, which are then hydrolysed by LPL and HL to form LDL (Nicoll and Lewis 1980). Since most of the TAG of the original VLDL particles has been hydrolysed, LDL particles have mainly CEs in their core. LDL has one apoB-100 attached to the surface, the same way as its parent particle VLDL (Hevonoja et al. 2000).

2.2.3.3 HDL

High density lipoproteins (HDLs) are secreted from the liver as discoidal nascent HDL in a process which requires ApoA-I binding to ATP-binding cassette transporter A1 (ABCA1) and budding of the plasma membrane (Phillips 2014, Francis 2016). ApoA-1 and ApoE, which are found on the surface of HDL, enable the detachment of the formed membrane structure (Francis 2016). The discoidal or pre-βHDL gathers CE through the function of LCAT, acquires a spherical shape and grows in size (Lund-Katz and Phillips 2010, Kuai et al. 2016). HDL can also receive cholesterol from the tissues though a scavenger receptor mediated uptake and exchange CE to TAG derived from other lipoproteins through the function of cholesterol ester transfer protein (CETP) (Bruce et al. 1998, Lund-Katz and Phillips 2010). Mature HDL can deliver its CE-rich cargo to the liver, and the whole process of HDL mediated CE delivery to the liver is termed reverse cholesterol transport (Lund-Katz and Phillips 2010). Importantly, HDL acts as an acceptor for cholesterol derived from macrophages in the walls of blood vessels, which promotes regression of atherosclerotic plaques thus inhibiting cardiovascular disease (Cuchel and Rader 2006).

2.2.3.4 Remodelling of lipoproteins

The lipids of lipoprotein particles are remodelled in the circulation by CETP and also phospholipid transfer protein (PLTP) (Tall 1995). CETP mediates the bidirectional transfer of CE and TAG between lipoproteins in plasma. It promotes the net mass transfer of CE synthesized in HDL into chylomicron and VLDL remnants and to LDL, and at the same time a net transfer of TAG happens in the opposite direction (Tall 1995, Bruce et al. 1998). CETP can also exchange phospholipids between lipoproteins, however, the net mass transfer of phospholipids occurs through the function of PLTP (Tall 1995, Bruce et al. 1998). PLTP transfers phospholipids between different HDL particles and between HDL and apoB-containing lipoproteins (Albers et al. 2012). When LPL hydrolyses lipoproteins, PLTP transfers the excess surface lipids to HDL (Albers et al. 2012).

Also several of the apolipoproteins on the surface of the lipoproteins can be exchanged in the circulation (McLeod and Yao 2016). The exchangeable lipoprotein ApoE, which is found on the surface of chylomicrons, VLDL and HDL (Frayn 2010), also increases CETP-mediated lipid exchange between lipoproteins (Kinoshita et al. 1993).

2.2.3.5 Lipoprotein uptake

Fatty acids released to circulation through hydrolysis of lipoproteins are taken up into tissues by the action of different transport and binding proteins (Eaton 2002), and correspondingly, lipoproteins are removed from the circulation by several types of receptors located on the surface of hepatocytes (Williams and Chen 2010, Pieper-Furst and Lammert 2013, Rohrl and Stangl 2013, Schneider 2016). Chylomicron remnants, LDL and VLDL particles are taken up via receptor-mediated endocytosis (Cooper 1997, Williams and Chen 2010, Schneider 2016). Members of the LDL receptor family bind ApoB-100 and ApoE-containing particles (Williams and Chen 2010, Pieper-Furst and Lammert 2013). Lipoprotein remnants can also be endocytosed by syndecan-1 heparan sulfate proteoglycan receptors, which bind ApoE, HL and LPL (Williams and Chen 2010). A third type of receptors, termed scavenger receptors, binds lipoproteins and a variety of other types of ligands they transport into cells (Zani et al. 2015). Scavenger receptor B1 is an HDL receptor, which has a crucial role in reverse cholesterol transport and cholesterol homeostasis as it transfers cholesterol esters from HDL into the liver (Rohrl and Stangl 2013). HDL can also be endocytosed and recycled upon scavenger receptor B1 mediated uptake (Silver et al. 2001). Scavenger receptors are expressed in several cell types and tissues, and many of them have been found to play a role in the development of atherosclerosis (Zani et al. 2015). Importantly, if LDL and chylomicron and VLDL remnants and are not removed from the circulation into the liver, they can be taken up into arterial walls causing atherosclerosis (Williams and Tabas 1995, Tabas et al. 2007, Khetarpal and Rader 2015). It has been shown that dietary 12-16 carbon-long saturated fatty acids reduce LDL receptor activity (Woollett et al. 1992), and diets rich in saturated fatty acids also increase the selective uptake of LDL CEs into the arterial wall (Seo et al. 2005).

Reversely, diets enriched in n-3 fatty acids decrease arterial LDL particle uptake and abolish the selective uptake of CE from LDL into the arterial walls (Chang et al. 2009).

2.2.4 β-oxidation

β-oxidation is a process in which fatty acids are broken down in order to produce energy in the citric acid cycle (Schulz 1991). In the liver, β-oxidation produces also ketone bodies, which are transported to other tissues via circulation to provide energy during fasting (Rui 2014). The fatty acids used for oxidation are released to circulation from adipose tissue during fasting when catecholamines induce the G protein- and cAMP-mediated activation of protein kinase A (Ahmadian et al. 2009). This leads to phosphorylation of perilipin altering its configuration and exposing the surface of the lipid droplet, which allows TAG hydrolysis by hormone-sensitive lipase, adipose triglyceride lipase, and monoacylglycerol lipase (Ahmadian et al. 2009). The latter two of the lipases also hydrolyse lipid droplets destined for β-oxidation in human hepatocytes (Quiroga and Lehner 2012). Moreover, fatty acids can be released for oxidation from lipoproteins by the action of LPL, HL as well as endothelial lipase (EL) (Schulz 1991, Wang et al. 2013, Olivecrona 2016). LPL hydrolyses mainly TAG, HL both TAG and phospholipids, and EL mainly phospholipids especially in HDL (Jaye et al. 1999, Santamarina-Fojo et al. 2004, Olivecrona 2016).

Fatty acids are taken up by the cells by three types of transport or binding proteins: fatty acid translocase (CD36; a B-type scavenger receptor), the plasma membrane fatty acid binding protein and the fatty acid transport proteins (Eaton 2002). This process is regulated at the transcriptional level by peroxisome proliferator-activated receptor (PPAR) γ (Rui 2014, Ipsen et al. 2018). In order to be oxidised in the mitochondria, the fatty acids need to be first activated by acyl-CoA synthetase and subsequently bound to carnitine to enable transportation by carnitine acyltransferases (CPT) I and II to the mitochondrial matrix where the fatty acids are again activated by binding to CoA and finally oxidized through a sequential removal of two-carbon units (Schulz 1991, Eaton 2002). Peroxisomal β-oxidation is needed for the initiation of the oxidation of polyunsaturated and very long-chain fatty acids; however its contribution to the total β-oxidation flux of long-chain fatty acids is likely no more than 10%, also in liver where peroxisomes are abundant (Eaton 2002). Increased expression or activity of PPARα promotes fatty acid β-oxidation in both mitochondria and peroxisomes (Rui 2014, Ipsen et al. 2018).

2.3 Cardiometabolic diseases

The term cardiometabolic diseases can be used for describing a group of conditions in which cardiovascular health is affected negatively by metabolic dysfunction. Obesity is a common risk factor for cardiometabolic diseases like type II diabetes, metabolic syndrome and the related NAFLD (James et al. 2004, Younossi et al. 2016, Emdin et al. 2017). NAFLD can be seen as the hepatic manifestation of the metabolic syndrome (Kotronen and Yki-Järvinen 2008, Vanni et al. 2010, Yki-Järvinen 2014). However, genetic NAFLD caused by PNPLA3^{I148M} and TM6SF2^{E167K} is not associated with the hallmarks of the metabolic syndrome like insulin resistance or dyslipidaemia, which is characterised by elevated plasma LDL and TAG-rich lipoproteins and reduced concentrations of HDL (Romeo et al. 2008, Speliotes et al. 2010, Kozlitina et al. 2014, Holmen et al. 2014). The TM6SF2^{E167K} even lowers plasma TAG and cholesterol (Holmen et al. 2014, Kozlitina et al. 2014). Nonetheless, obesity amplifies the effect of the predisposing genetic variants, further increasing the risk of developing genetic NAFLD (Stender et al. 2017). Type 2 diabetes and metabolic syndrome are both risk factors for cardiovascular disease (Wilson et al. 2005, Einarson et al. 2018), whose main pathological process is the formation of a cholesterol-rich atherosclerotic plaque in the arterial wall (Bentzon et al. 2014). Naturally occurring LOF variants of ANGPTL3 reduce the concentration of circulating cholesterol and TAG carried in lipoproteins (Musunuru et al. 2010, Minicocci et al. 2012, Stitziel et al. 2017), which has made ANGPTL3 inhibition an attractive possibility for treatment of atherosclerosis (Dewey et al. 2017, Graham et al. 2017). **Figure 3** shows the relation between cardiometabolic diseases and the genetic variants studied in this thesis project.

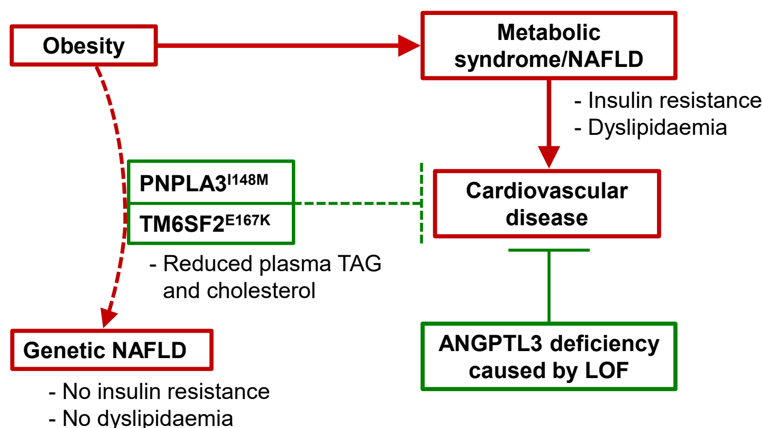

Figure 3. The adverse and protective effects of PNPLA3^{I148M}, TM6SF2^{E167K} and ANGPTL3 loss-of-function (LOF) on cardiometabolic diseases. Negative effects are depicted using red arrows and positive outcomes using green lines.

2.3.1 NAFLD

NAFLD is defined by the presence of steatosis (i.e. TAG accumulation) in more than 5 % of hepatocytes, which is not due to secondary causes or excess alcohol consumption (Cohen et al. 2011, European Association for the Study of the Liver (EASL) et al. 2016). NAFLD is the most common liver disease in the world and its prevalence is currently 25 % in the adult population, varying between 32 % in the Middle East and 13 % in Africa (Younossi et al. 2016). In severely obese individuals the prevalence of NAFLD is 90 % and in patients with type 2 diabetes 76 % (Younossi et al. 2016). However NAFLD has also been reported to affect more than 10 % of lean individuals in several Asian populations, India having the highest rate with 20 % (Wattacheril and Sanyal 2016). Up to 30 % of patients with simple hepatic steatosis develop non-alcoholic steatohepatitis (NASH) in which there is already clear hepatocyte injury, cell death, inflammation and fibrosis in the liver (Cohen et al. 2011, Younossi et al. 2016). NASH in turn develops into advanced fibrosis in 40 % of patients (Younossi et al. 2016), and the most severe outcome of the disease is hepatocellular carcinoma, the risk of which is higher in patients with obesity or type 2 diabetes (Yu et al. 2013).

For hepatic steatosis to develop, there needs to be an imbalance between the storage and removal of fatty acids and TAG; that is the rate of TAG synthesis needs to be greater than the rate of β-oxidation and VLDL secretion (Cohen et al. 2011, Ipsen et al. 2018). It has been shown by stable isotope studies that increased fatty acid flux from the adipose tissue and fatty acid *de novo* synthesis are the main mechanisms contributing to hepatic fat accumulation in NAFLD patients (Donnelly et al. 2005). Obesity-related or metabolic NAFLD is associated with insulin resistance (Kotronen and Yki-Järvinen 2008, Yki-Järvinen and Luukkonen 2015), which in the adipose tissue leads to increased lipolysis and release of fatty acids into the circulation (Vanni et al. 2010). In a healthy liver, insulin inhibits glucose production between meals and normal blood glucose levels are maintained. When insulin resistance develops, this balance is disturbed leading to increased glucose production and subsequently increased insulin secretion (Vanni et al. 2010). Hepatic insulin resistance also leads to increased secretion of large VLDL particles and thereby generation of atherogenic small dense LDL particles through the function of CETP and hepatic lipase (Adiels et al. 2008, Tchernof and Despres 2013, Brouwers et al. 2019). This same process leads to formation of easily degraded small dense HDL particles thus lowering circulating HDL (Rashid et al. 2003).

The mechanisms underlying the progression of NAFLD to NASH are yet to be elucidated, but also genetic predisposition is known to play a role in the process (Petta 2009, Rotman et al. 2010, Speliotes et al. 2010, Liu et al. 2014, Ioannou 2016, Pingitore et al. 2016). Free cholesterol and free fatty acid mediated lipotoxicity and subsequent pro-inflammatory cytokine production and oxidative stress have been suggested to be behind the inflammatory and fibrotic processes of NASH (Petta 2009, Vanni et al. 2010, Ioannou 2016).

2.3.2 Genetic NAFLD

During the last decade genome-wide association studies have revealed several gene variants that increase the risk of developing NAFLD (Anstee and Day 2015, Eslam and George 2020). Two of these, PNPLA3^{I148M} and TM6SF2^{E167K} will be discussed in detail.

2.3.2.1 PNPLA3 and its I148M variant

In 2008 Romeo *et al.* (2008) described a single nucleotide polymorphism in the PNPLA3 gene (rs738409; C→G at position 148 of the gene leading to substitution of isoleucine to methionine, I148M), which is strongly associated with NAFLD. In this original study the genetic background was found to affect the frequency of the variant allele, the two ends being African Americans, of whom 17 % were carrying at least one copy of the variant allele, and Hispanics, of whom 49 % had the variant allele. The association between PNPLA3^{I148M} and NAFLD has since been shown in several different studies and in different ethnic groups (Chen et al. 2015). PNPLA3^{I148M} is also significantly associated with the development of NASH, fibrogenesis and the severity of liver fibrosis in NAFLD patients (Rotman et al. 2010, Valenti et al. 2010, Speliotes et al. 2010, Krawczyk et al. 2011), also in pediatric NAFDL (Valenti and Alisi et al. 2010). The effect of PNPLA3^{I148M} is dose dependent meaning that the individuals homozygous for the PNPLA3^{I148M} variant have an even higher risk for developing NAFLD and for the progression of the disease compared to heterozygous subjects (Romeo et al. 2008, Valenti and Al-Serri et al. 2010).

PNPLA3^{I148M} is not associated with insulin resistance or dyslipidaemia (Romeo et al. 2008, Kantartzis et al. 2009, Speliotes et al. 2010) and it causes a more metabolically benign NAFLD. In a study by Kantartzis *et al*. (2009) insulin sensitivity was shown to be higher in NAFLD patients carrying the PNPLA3^{I148M} allele than in NAFDL patients with no variant allele, and there was no statistically significant difference between the insulin sensitivity of healthy control subjects and NAFLD patients homozygous for PNPLA3^{I148M} variant allele. In the same study, obese subjects carrying the variant allele had higher insulin sensitivity than control subjects, when adjusted for age, sex, total fat, visceral fat, and liver fat. In genetic screening studies using large cohorts, PNPLA3^{I148M} variant allele has been shown to protect from coronary artery disease (Liu et al. 2017, Simons et al. 2017).

Although the association of PNPLA3^{I148M} with NAFLD is well established, the mechanism of PNPLA3 function has remained unclear. In humans, PNPLA3 is expressed mainly in the liver but also in the adipose tissue and skin (Huang et al. 2010). During fasting the expression level is low but is rapidly increased after a carbohydrate meal (Lake et al. 2005, Huang et al. 2010, Rae-Whitcombe et al. 2010), likely due to insulin mediated activation of SREBP (Huang et al. 2010, Qiao et al. 2011, Soronen et al. 2012) and also through insulin-independent activation of ChREBP (Dubuquoy et al. 2011, Perttilä et al. 2012). PNPLA3 localizes to lipid droplets, and overexpression of PNPLA3^{I148M} increases their size (He et al. 2010, Chamoun et al. 2013). Chamoun *et al.* (2013) also suggested that PNPLA3 may play a role in lipid

droplet formation. It has been shown *in vitro* that wild type PNPLA3 (PNPLA3^{WT}) but not PNPLA3^{I148M} hydrolyses emulsified TAG (Jenkins et al. 2004, Lake et al. 2005, He et al. 2010). In addition, PNPLA3^{WT} has acylglycerol transacylase activity (Jenkins et al. 2004). The preferred substrate of the protein is oleic acid (Huang et al. 2011), and it has been proposed that the amino acid substitution in PNPLA3^{I148M} changes the catalytic triad of the protein thus abolishing its hydrolase activity (He et al. 2010). Kumari et al. (2012) claimed that PNPLA3 is a lysophosphatidic acid acyltransferase and PNPLA3^{I148M} would function more efficently in this activity. Then again Pirazzi et al. (2012) suggested that the PNPLA3^{I148M} related NAFLD would be a consequence of reduced VLDL lipidation and secretion. However, they also speculated that the role of PNPLA3 in this process could be related to intracellular TAG synthesis or the remodelling of lipid droplets. Studies using a PNPLA3^{I148M} overexpressing mouse model support the remodelling theory, as both impaired hydrolysis of TAG and a relative depletion of long-chain PUFA-containing TAGs was noticed in these mice (Li et al. 2012). During the last two years, more evidence has emerged to support the remodelling function of PNPLA3 and its role related to lipid droplet hydrolysis (BasuRay et al. 2017, Mitsche et al. 2018, Wang et al. 2019, Negoita et al. 2019, Luukkonen et al. 2019). These findings will be addressed further in relation to publication I in the Results and discussion section.

2.3.2.2 TM6SF2 and its E167K variant

A genetic variant in the TM6SF2 gene (rs58542926, A→G at position 167 leading to substitution of glutamic acid to lysine, E167K) was found to be associated with NAFLD in 2014 in two separate studies (Kozlitina et al. 2014, Holmen et al. 2014). Based on the original study by Kozlitina *et al.* (2014) genetic background also affects the frequency of TM6SF2^{E167K}, which is approximately 7 % in individuals of European ancestry and 3 % in African Americans. They also suggested that TM6SF2^{E167K} is a misfolded protein and therefore readily degraded. This decreased stability caused by the amino acid substitution has later been confirmed by others (Ehrhardt et al. 2017).

TM6SF2 is a membrane protein predominantly expressed in the liver and small intestine and it localizes to the ER and Golgi complex of hepatocytes (Mahdessian et al. 2014, Kozlitina et al. 2014, Smagris et al. 2016). Accordingly, TM6SF2 has been suggested to play a role in VLDL secretion (Mahdessian et al. 2014, Kozlitina et al. 2014, Ehrhardt et al. 2017) and lipidation (Smagris et al. 2016). This would also explain why reduced levels of TM6SF2 caused by the destabilizing E167K variant would lead to hepatic lipid accumulation as neutral lipids are not secreted and remain in the liver. Mahdessian *et al.* (2014) saw clearly reduced TAG secretion but only a modest reduction in the secretion of ApoB due to TM6SF2 inhibition in human hepatocytes. Hepatic 3D spheroid and human data also point towards reduced ApoB secretion due to TM6SF2^{E167K} (Kim et al. 2017, Prill et al. 2019). In contrast, in mice lacking *Tm6sf2* a reduced secretion rate of VLDL TAG was noticed without reduction of secreted ApoB but with a reduction in secreted VLDL particle size and plasma cholesterol levels (Smagris et al. 2016). In another study also executed with *Tm6sf2*-knockout mice,

decreased levels of plasma total and LDL-cholesterol were noticed and it was also reported that liver-specific expression of TM6SF2 affects several genes regulating cholesterol metabolism, therefore making TM6SF2 a possible target for treating cardiovascular disease (Fan et al. 2016).

Indeed, TM6SF2^{E167} seems to protect from myocardial infarction (Holmen et al. 2014, Dongiovanni et al. 2015, Simons et al. 2017, Li et al. 2018), but at the same time it causes NAFLD with increased risk of progression into NASH and hepatic fibrosis or cirrhosis (Liu et al. 2014, Dongiovanni et al. 2015). However, NAFLD induced by TM6SF2^{E167} is not associated with insulin resistance or dyslipidaemia (Kozlitina et al. 2014, Zhou et al. 2015), and the more progression prone NAFLD may be explained by increased ER stress caused by TM6SF2^{E167} (O'Hare et al. 2017). In discordance with earlier findings, it has been reported that, in addition to the lack of TM6SF2 caused by the E167K variant, also increased expression of hepatic TM6SF2 could lead to the same anti-atherogenic and pro-NAFLD phenotype (Ehrhardt et al. 2017). Based on studies using cultured human enterocytes and larval zebrafish, TM6SF2 may also play a role in intestinal lipid and ER homeostasis (O'Hare et al. 2017). As the current knowledge on the function of TM6SF2 and its NAFLD causing variant is somewhat contradictory, more information on their mechanisms of function is still required.

2.3.3 Atherosclerosis

Atherosclerosis is a key pathological process in cardiovascular diseases. It is a condition in which an artery becomes narrowed due to the development of a cholesterol-enriched lesion, or atherosclerotic plaque, in the arterial intima (Williams and Tabas 1995, Tabas et al. 2007). Rupturing of the plaque and the resulting thrombus formation may cause occlusion of the artery leading to for example myocardial infarction or stroke (Bentzon et al. 2014). The development of an atherosclerotic plaque begins when ApoB-containing lipoproteins cross the endothelium and are retained in the arterial intima (Tabas et al. 2007, Bentzon et al. 2014). The retention is mediated by proteoglycans of the subendothelial extracellular matrix (Skalen et al. 2002) and the trapped lipoproteins are modified so that they aggregate and become oxidized (Pentikäinen et al. 2000, Steinberg 2009). This leads to an inflammatory process in which monocytes enter the intima, turn into macrophages that take up the modified lipoproteins mainly via scavenger receptors, and turn into foam cells (Steinberg 2009, Zani et al. 2015, Chistiakov et al. 2016). The inflammation process is intensified by the entry of other inflammatory cells and the retention of lipoproteins increases further (Pentikäinen et al. 2000, Tabas et al. 2007, Bäck et al. 2019). Smooth muscle cells form a fibrous cap over the lesion, but as the foam cells die and the core of the cholesterol-enriched lesion becomes necrotic, the plaque becomes more unstable and the fibrous cap more prone to rupture (Bentzon et al. 2014).

Although there are conditions like familial hypercholesterolemia, in which a substantially elevated concentration of circulating LDL is the primary reason for development of an

atherosclerotic lesion (Wiegman et al. 2015), atherosclerosis is considered as a multifactorial disease. Thus elevated LDL cholesterol or dyslipidaemia together with other risk factors like hypertension, obesity, metabolic syndrome and diabetes is in most cases causing the disease (Berenson et al. 1998, Fruchart et al. 2004). There are also more recently found factors such as elevated levels of circulating triglyceride-rich lipoprotein remnants, small dense LDL and lipoprotein(a), which contribute to the disease risk (Ridker et al. 2001, Fruchart et al. 2004, Khetarpal and Rader 2015). Lowering LDL levels by drugs such as statins, ezetimibe and proprotein convertase subtilisin kexin type 9 (PCSK9) inhibitors has been a successful strategy to combat atherosclerosis, however there is still a need for new approaches in order to reduce the residual risk (Shapiro and Fazio 2016, Kersten 2017, Gaudet et al. 2017, Hegele and Tsimikas 2019).

2.3.3.1 ANGPTL3 and its loss-of-function variants

ANGPTL3 is a protein synthesized and secreted mainly by the liver (Conklin et al. 1999), and it circulates in plasma inhibiting LPL and EL activity (Shimizugawa et al. 2002, Shimamura et al. 2007). ANGPTL3, like most of the members of the ANGPTL family, possesses a signal sequence in the amino-terminus, a coiled-coil domain and a fibrinogen-like domain (Zhang and Abou-Samra 2013). In addition, ANGPLT3 has a specific region binding to LPL and it mediates LPL inactivation by enhancing the cleavage of the lipase by proprotein convertases (Liu et al. 2010, Zhang and Abou-Samra 2013). During this process, LPL also dissociates from the cell surface (Liu et al. 2010). A heparin-binding site located in the amino-terminal domain of ANGPTL3 most probably mediates the inhibition of EL by ANGPLT3 (Shimamura et al. 2007). ANGPTL3 seems to work in concert with ANGPTL8, which is lacking the fibrinogen-like domain (Zhang and Abou-Samra 2013). ANGTL3 may be more potent in the presence of ANGPTL8, and ANGPTL8 likely needs ANGPTL3 to be able to inhibit LPL (Quagliarini et al. 2012, Haller et al. 2017). In mice, ANGPTL3 has also been shown to activate lipolysis and to stimulate the release of free fatty acids and glycerol from adipocytes (Shimamura et al. 2003), but also to promote the uptake of VLDL-TAG derived fatty acids into white adipose tissue after feeding (Wang and McNutt et al. 2015). In the liver, hepatocytes are solely responsible for the production of ANGPTL3 (Kersten 2017). Mouse studies suggest that *ANGPTL3* expression does not change significantly after a meal or during fasting (Ge et al. 2005), however, in human hepatocytes insulin decreases *ANGPTL3* expression and secretion (Nidhina Haridas et al. 2015). *ANGPTL8* expression levels are reduced during fasting but restored after a meal in the liver and adipose tissue of both humans and mice (Quagliarini et al. 2012). On the contrary, *ANGPTL4*, another inhibitor of LPL belonging to the same protein family, is induced by fasting in both the liver and adipose tissue (Ge et al. 2005).

ANGPTL3 LOF was first described in mice in 2002 (Koishi et al. 2002), and later in human subjects with extremely low plasma levels of TAG and LDL and HDL cholesterol, a condition termed familial combined hypolipidaemia (Musunuru et al. 2010). Several different ANGPLT3 LOF mutations have been found in humans (Arca et al. 2013, Kersten 2017,

Dewey et al. 2017). Individuals homozygous for ANGPLT3 LOF lack circulating ANGPTL3 and have increased LPL activity, low plasma levels of VLDL, LDL and HDL, increased insulin sensitivity and decreased serum free fatty acids (Minicocci et al. 2012, Robciuc et al. 2013, Arca et al. 2013). A recent study showed that ANGPTL3 deficiency leads to reduction of the proportion of cholesterol in triglyceride-rich lipoproteins and their remnants (Tikkanen et al. 2019). ANGPTL3 LOF carriers may also have enhanced hepatic fatty acid β-oxidation as hinted by an elevated ketone body production (Tikkanen et al. 2019). In mice, inactivating or silencing ANGPTL3 reduces hepatic VLDL-TAG secretion and enhances the uptake of ApoB-containing lipoproteins by the liver (Wang and Gusarova et al. 2015, Xu et al. 2018).

No adverse effects have been reported of ANGPTL3 LOF in humans, and importantly, ANGPLT3 deficiency has been found to protect from atherosclerotic cardiovascular disease (Minicocci et al. 2012, Minicocci et al. 2013, Dewey et al. 2017, Stitziel et al. 2017). Also subjects heterozygous for ANGPTL3 LOF have a reduced risk of coronary artery disease, even though there is only a modest drop in their plasma TAG and LDL-cholesterol compared to homozygous subjects (Dewey et al. 2017, Stitziel et al. 2017). For these reasons, ANGPTL3 is a promising target for treating cardiovascular disease and clinical trials are already ongoing. Evinacumab, a monoclonal antibody against ANGPTL3, reduced fasting plasma TAG levels up to 80 % and LDL-cholesterol up to 23 % in a dose-dependent manner (Dewey et al. 2017, Ahmad et al. 2019). The LDL-cholesterol lowering mechanism of evinacumab is independent of the LDL receptor and thus also patients with familial hypercholesterolemia have been shown to substantially benefit from the treatment as their LDL-cholesterol has been reduced by one half (Gaudet et al. 2017, Banerjee et al. 2019). In dyslipideamic mice, evinacumab reduced plasma levels of TAG and LDL- and HDL-cholesterol without changing the TAG-content of the liver, adipose tissue, or heart (Gusarova et al. 2015). Also the area of atherosclerotic lesions and their necrotic content was shown to be reduced by the antibody treatment in mice having dyslipidaemia (Dewey et al. 2017). Another ANGPLT3 lowering treatment with antisense oligonucleotides that inhibit hepatic ANGPLT3 production has yielded similar results to those seen with the monoclonal antibody approach. In humans, the antisense oligonucleotide treatment reduced the levels of atherogenic lipoproteins, and in mice it also slowed the progression of atherosclerosis (Graham et al. 2017). Even though these clinical trials have had successful outcomes, the function of hepatic ANGPTL3 and how its depletion may affect hepatocytes remains unclear. Also the effect of ANGPTL3 deficiency on the detailed lipid composition of lipoproteins and the possible contribution of the altered lipid profile to the protection from cardiovascular disease has not been studied until now.

3 AIMS OF THE STUDY

1. To elucidate the role of PNPLA3 in the lipid metabolism of human hepatocytes and the connection between the PNPLA3 I148M variant and increased liver fat content. (I)

2. To investigate the function of TM6SF2 in hepatic lipid metabolism and how TM6SF2 deficiency causes fat accumulation in the liver. (II)

3. To study how ANGPTL3 depletion affects hepatic lipid metabolism and how it is reflected in the circulating lipoproteins. (III)

4 MATERIALS AND METHODS

Methods performed by the author to complete the thesis work are listed in **Table 1**, and the workflows of lipidomics experiments are depicted in **Figure 4**. Further descriptions and a full listing of the materials and methods used in the thesis project can be found in publications I-III.

Table 1. Summary of methods used by the author.

Method	Publication
Cell culture	I-III
Gene overexpression (transfection)	I
ShRNA lentiviral transduction	II-III
Labelling studies	
- stable isotopes (→ ESI-MS/MS)	I-II
- radioactive isotopes (→ liquid scintillation counting)	II-III
BCA protein assay total protein quantification	I-III
Folch lipid extraction	I-III
Bligh and Dyer lipid extraction	I
Fatty acid methyl ester preparation	I-III
Mass spectrometry	
- ESI-MS	I
- ESI-MS/MS	I-III
Gas chromatography	
- GC-FID	I-III
- GC-MSD	II-III
RNA extraction	II-III
Gene expression analysis (qPCR)	II-III
Mitochondrial oxygen consumption rate measurement (Seahorse extracellular flux analysis)	II
ELISA	II
One-way ANOVA & Newman-Keuls test of means	I
Two-tailed Student's t-test	II-III
Principal component analysis (PCA) & soft independent modeling of class analogy (SIMCA)	I-III

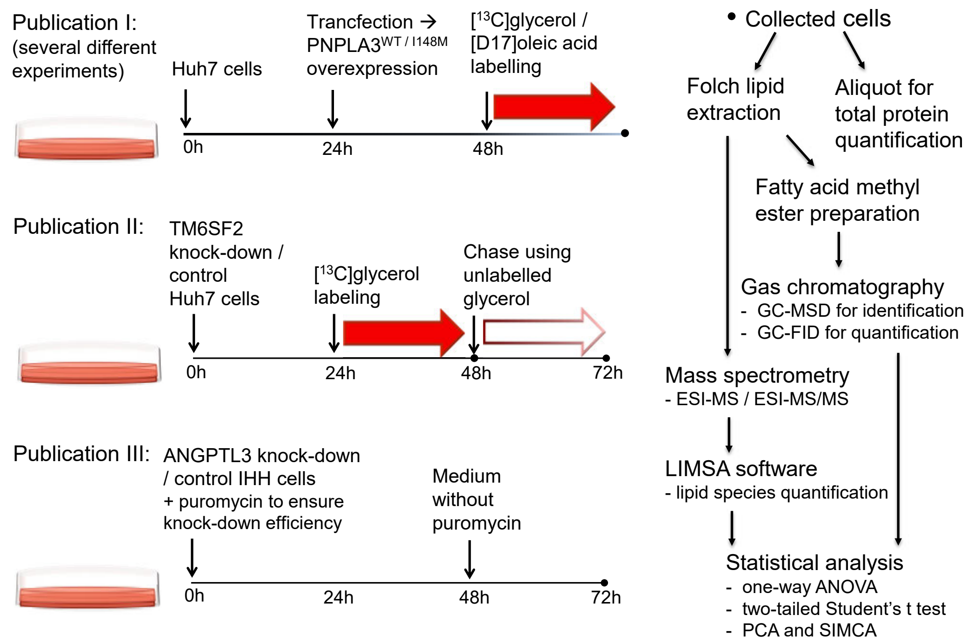

Figure 4. Workflow of the performed lipidomics experiments. GC-MSD, GC-FID=gas chromatography coupled to mass spectrometry/flame ionization detector; ESI-MS, ESI-MS/MS= electrospray ionization mass spectrometry/triple quadrupole mass spectrometry; LIMSA=Lipid Mass Spectrum Analysis software (Haimi et al. 2006); ANOVA=analysis of variance; PCA=Principal component analysis; SIMCA=soft independent modeling of class analogy.

5 RESULTS AND DISCUSSION

5.1 PNPLA3 functions as a remodelling protein and the I148M variant shows reduced remodelling activity (I)

5.1.1 PNPLA3^{I148M} overexpression causes net accumulation of TAG in hepatocytes

The function of PNPLA3^{WT} and the effect of PNPLA3^{I148M} were studied in HuH7 human hepatoma cells overexpressing either form of the protein. These were compared with control cells transfected with an empty plasmid vector, and the expression was confirmed by Western blot analysis (**I, Fig 1A**). We used [^{13}C]glycerol labelling of the cells followed by electrospray ionization mass spectrometry (ESI-MS) to get a detailed view of the lipid metabolism. When looking at the total amount of TAG after a 24-hour labelling period, there was a statistically significant difference between the cells; PNPLA3^{I148M} overexpressing cells had an increased level of TAG when compared to PNPLA3^{WT} and control cells (**Figure 5A**). However, the difference was due to increased amount of unlabelled TAG and there was no difference between the groups in the total amount of newly synthesized [^{13}C]glycerol labelled TAG (**Figure 5B,C**). If PNPLA3 would function primarily as a TAG lipase (He et al. 2010, Huang et al. 2011), one should expect to see a drop in the level of TAG upon PNPLA3^{WT} overexpression. Thus our data does not support the view of PNPLA3 being a mere lipase, nor does it point towards a simple lipogenic function of PNPLA3 (Kumari et al. 2012), since *de novo* synthesis of TAG was not increased (**Figure 5C**). This is consistent with a previous finding in the same cell model and setting, where *de novo* lipogenesis was not significantly affected in PNPLA3^{WT} or PNPLA3^{I148M} overexpressing cells when compared to control cells in normal cell culture conditions as measured by [^{3}H]acetic acid labelling (Perttilä et al. 2012). In addition, we did not observe any difference between the groups in the total amount of TAG precursors PA and DAG, nor in the amount of PC (**I, Fig. 2 B-D, inserts**). These results regarding the lipid levels are also consistent with human data on PNPLA3^{I148M} variant and PNPLA3^{WT} carriers (Peter et al. 2014). In line with earlier findings (He et al. 2010, Perttilä et al. 2012), we saw a delay in TAG hydrolysis in the PNPLA3^{I148M} overexpressing cells when the transfected cells were cultured for 6 or 24 hours in a medium supplemented with 5 % foetal bovine serum and Triacsin C, which is a long chain fatty acyl-CoA synthetase inhibitor (Omura et al. 1986, Igal et al. 1997).

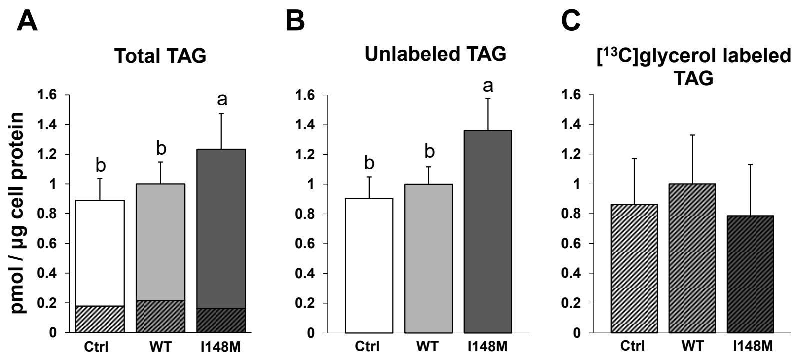

Figure 5. Overexpression of PNPLA3^{148M} in HuH7 cells induces net TAG accumulation but does not affect newly synthesized TAGs. (A) Total TAGs after 24-hour [^{13}C]glycerol labelling analysed by electrospray ionization mass spectrometry (ESI-MS) (B) Unlabelled TAGs after 24-hour [^{13}C]glycerol labelling. (C) Newly synthesized [^{13}C]glycerol labelled TAGs after 24-hour labelling. Values from two separate experiments were normalized by setting PNPLA3^{WT} to 1. The means with no common letter differ at p<0.05 level (one-way ANOVA followed by Newman-Keuls test of means). Error bars, SD; n=7. Ctrl=control, WT=wild type PNPLA3; I148M=PNPLA3 I148M variant. Adapted from Publication I, Ruhanen *et al.* 2014.

5.1.2 PNPLA3^{WT} participates in TAG remodelling more efficiently than PNPLA3^{I148M}

To get a more precise picture of the effect of PNPLA3^{WT} and PNPLA3^{I148M} on hepatic lipid metabolism, we subjected the lipid species profiles of the [^{13}C]glycerol labelled cells to principal component analysis (PCA). The PCA of the TAG composition of the cells showed a clear and also statistically significant (p<0.05 in soft independent modelling of class analogy (SIMCA) analysis) separation of all groups (**Figure 6A**). The principal component axes PC1 and PC2 appeared to separate the groups based on the degree of fatty acid unsaturation and the presence of the [^{13}C]glycerol label, respectively. The control cells contained relatively more TAG species whose acyl chains had several double bonds, whereas the PNPLA3^{WT} and PNPLA3^{I148M} overexpressing cells contained TAG enriched in saturated and monounsaturated fatty acids (SFAs and MUFAs). In PNPLA3^{WT} cells the SFA- and MUFA-containing TAGs were largely [^{13}C]glycerol labelled while the PNPLA3^{I148M} cells were enriched in equivalent unlabelled TAGs. PCA of the species profile of unlabelled DAGs showed similarity to the TAG biplot in that the control cells were enriched in PUFA-containing DAGs and PNPLA3^{I148M} overexpressing cells possessed more DAGs having SFA and MUFA moieties (**I, Fig 2B**). These cells also differed from each other statistically significantly in SIMCA analysis. The PNPLA3^{WT} cells did not differ from the control cells according to SIMCA and in the PCA they showed an intermediate profile between the other two groups. Furthermore, when examining a PCA biplot of the composition of unlabelled PC species, the patterns of the groups were the opposite than found for TAG; the control cells were enriched in SFA- and MUFA-containing PCs and the PNPLA3^{WT} and PNPLA3^{I148M} overexpressing cells contained more PCs with PUFA moieties, and especially 20:4n-6 (for example in species 36:4, 38:4 and 38:5) (**Figure 6B**). Importantly, this finding was more prominent in the PNPLA3^{WT} cells. Both the PNPLA3^{WT} and PNPLA3^{I148M} differed from the control cells according to SIMCA (p<0.05). In human data, relative depletion of fatty acids 20:3n-6, 20:4n-6, 22:4n-6 and 22:5n-

6 in the elevated liver TAGs of PNPLA3^{I148M} carriers has been reported (Peter et al. 2014), but on the other hand PUFA-containing TAGs have been shown to accumulate in the livers of PNPLA3^{I148M} carriers compared to noncarriers (Luukkonen et al. 2016). In human PNPLA^{I148M} overexpressing mice, there was a relative depletion of PUFAs in hepatic TAGs (Li et al. 2012). Similarly, in PNPLA3^{I148M} knock-in mice, very long chain PUFAs were depleted from TAG and enriched in phospholipids, but conversely, in mice completely lacking Pnpla3 or having a catalytically inactive version of the protein, very long chain PUFAs were enriched in TAG and depleted from phospholipids (Mitsche et al. 2018).

Our findings are compatible with a TAG remodelling activity of PNPLA3, according to which PNPLA3 would participate in transferring fatty acids, from TAG to membrane phospholipids like PC, and that the PNPLA3^{I148M} amino acid substitution leads to a LOF hindering this remodelling activity. We confirmed our remodelling hypothesis in an experiment in which we applied stable isotope [D17]18:1n-9 labelling to our cell model. During 24-hour labelling PNPLA3^{WT} overexpressing cells incorporated more label into their TAGs compared to control and PNPLA3^{I148M} cells (**I, Fig. 3**). During the following 48 hours the relative amount of label also decreased faster in the PNPLA3^{WT} cells. Thus, PNPLA3^{WT} overexpression enhanced both the incorporation into and removal of fatty acids from TAGs, whereas PNPLA3^{I148M} overexpressing cells behaved similarly to control cells. The noticed TAG remodelling activity of PNPLA3 could be mediated though a TAG lipase or transacylase activity of the protein (Jenkins et al. 2004, Lake et al. 2005, He et al. 2010). PNPLA3 has been reported to have a strong preference for oleic acid (Huang et al. 2011), and thus we tested the remodelling activity using a labelled form of this fatty acid. However, recent studies have suggested that PUFAs may be more relevant in the context of the remodelling activity of PNPLA3 (Mitsche et al. 2018, Luukkonen et al. 2019). Our PCA data are compatible with a PUFA-specific remodelling activity of PNPLA3, since PCs of the PNPLA3^{WT} cells showed a more prominent enrichment of PUFAs than PNPLA3^{I148M} (**Figure 6B**).

We were the first to report the remodelling activity of PNPLA3, and others have later confirmed this finding. Mitsche (2018) used both knock-in and knock-out mouse models to show that PNPLA3 transfers very long-chain PUFAs from TAGs to phospholipids in lipid droplets. Luukkonen et al. (2019) utilized labelled PUFAs and SFAs to study the processing of fatty acids in human subjects homozygous for PNPLA3^{WT} or PNPLA3^{I148M} and also in cells homozygous for PNPLA3^{WT}, PNPLA3^{I148M} or PNPLA3 deletion. They came to a conclusion that PNPLA3^{I148M} would be a LOF allele defective in remodelling hepatic TAGs. They suggested that PNPLA3 is a PUFA-specific transacylase or a PUFA-specific lipase and that PNPLA3 would promote the transfer of PUFAs from DAGs to generate PCs enriched in PUFA. This remodelling model also explains their previous finding of TAGs being enriched in PUFAs in carriers of the PNPLA3^{I148M} variant compared with noncarriers (Luukkonen et al. 2016). In the same study they showed that in the metabolic NAFLD, which is associated with insulin resistance, the hepatic lipid profile is the opposite, that is, SFAs are enriched in TAGs (Luukkonen et al. 2016). They further contemplated that retention of PUFA-containing TAGs in the liver could provide an explanation why PNPLA3^{I148M} carriers are protected against cardiovascular disease despite having a fatty liver (Liu et al. 2017, Simons et al. 2017).

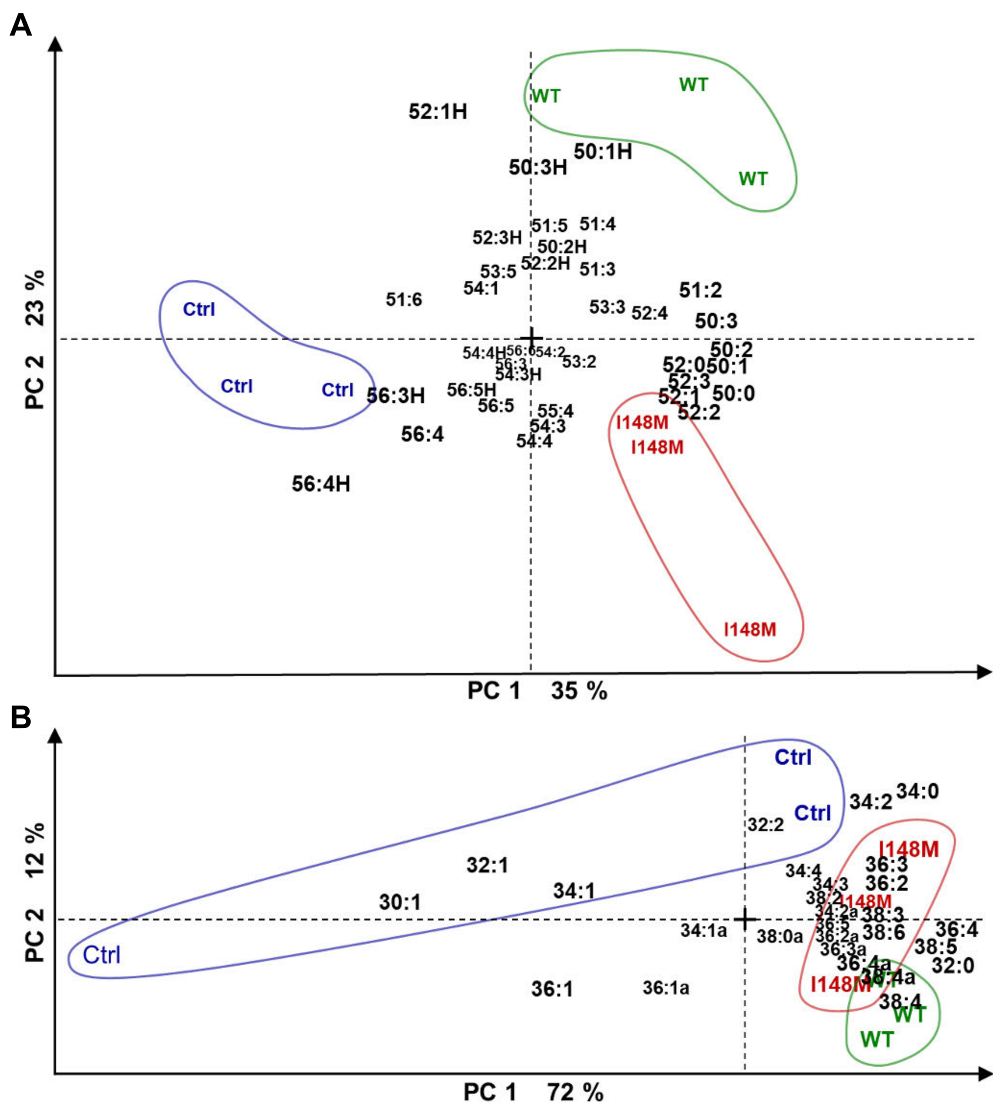

Figure 6. PCA shows differences in the lipid composition of PNPLA3^{WT} or PNPLA3^{I148M} overexpressing HuH7 cells when compared to control cells. The arrows represent the directions of the two principal components (PC1 and PC2) and the percentages show the proportion of the data variation each axis explains. The origin of the PCA biplot is marked with + and samples located furthest from it on one side contain relatively more of the lipid species furthest on that same side. (A) PCA of TAG species after 24-hour [^{13}C]glycerol labeling. Species present at >0.5 mol% were used as variables. Lipid species markings: 56:3H=56 carbons and 3 double bonds in the acyl chains, H=heavier *i.e.* [^{13}C]glycerol labelled species; Ctrl=control, WT=wild type PNPLA3; I148M=PNPLA3 I148M variant. (B) PCA of PC species. Species present with >0.5 mol% were used as variables; a=alkyl-acyl species (instead of diacyl species). Adapted from Publication I, Ruhanen *et al.* 2014.

When TAGs are hydrolysed to DAGs and then re-esterified, some released fatty acids are utilized in other processes such as oxidation, phospholipid synthesis and VLDL secretion (Lankester et al. 1998). Thus a remodelling defect of PNPLA3^{I148M} could lead to a gradual TAG accumulation as the remodelling cycle is slowed down. There is also evidence that PNPLA3^{I148M} impairs lipid droplet hydrolysis. We showed that PNPLA3^{I148M} localizes more extensively to the surface of a lipid droplet than PNPLA3^{WT}, and that fatty acid loading leading to enlarged lipid droplets increases the association of both forms of the protein with the lipid droplet (**I, Fig. 4**). It has been since shown in PNPLA3^{I148M} knock-in mice that increased liver fat is associated with PNPLA3^{I148M} accumulation on hepatic lipid droplets (Smagris et al. 2015). It was later proposed that PNPLA3^{I148M} disrupts ubiquitylation and proteasomal degradation of the protein, leading to accumulation of PNPLA3^{I148M} and impaired mobilization of TAG from lipid droplets (BasuRay et al. 2017). It was also recently suggested that PNPLA3^{I148M} could promote hepatic lipid accumulation by restricting the access of CGI-58, an activator of triglyceride hydrolases (Oberer et al. 2011), to adipose triglyceride lipase (Wang et al. 2019). In addition, PNPLA3^{I148M} has been shown to localize on lipid droplets that resist starvation-mediated degradation possibly by inhibiting autophagosome formation (Negoita et al. 2019). Thereby the reduced autophagy of hepatic lipid droplets caused by the PNPLA3^{I148M} presents another conceivable mechanism leading to hepatic steatosis in the variant carriers.

The n-3 PUFAs, especially 22:6n-3 and 20:5n-3, have been shown to be effective in treatment of NAFLD (Scorletti et al. 2014). However, n-3 PUFA treatment does not appear to be equally successful in all patient groups. In fact, NAFLD patients homozygous for PNPLA3^{I148M} had higher liver fat percentage after taking a 4 g daily 22:6n-3+20:5n-3 supplement for 15–18 months than before the trial (Scorletti et al. 2015). They also displayed decreased 22:6n-3 enrichment in erythrocyte membranes, which is an important finding since erythrocyte 22:6n-3 enrichment after n-3 PUFA supplementation has been shown to be linearly associated with decreased liver fat percentage (Scorletti et al. 2014). These findings concerning the treatment response of PNPLA3^{I148M} carriers homozygous for the allele are plausible in the light of the remodelling function of PNPLA3. PNPLA3 transfers PUFAs from TAGs to PCs in hepatic lipid droplets, and since the PNPLA3^{I148M} LOF variant carriers show accumulation of PUFAs in hepatic TAGs (Luukkonen et al. 2016) liver fat accumulation after dietary supplementation of PUFAs is not surprising. In addition, the dietary n-6/n-3 PUFA ratio seems to play a part in defining the strength of effect of PNPLA3^{I148M} in NAFLD patients, since in a paediatric obese population, an association between a high dietary n-6/n-3 PUFA ratio and liver fat content as well as liver damage was seen in subjects homozygous for PNPLA3^{I148M} (Santoro et al. 2012). Therefore the effects of different fatty acids on PNPLA3^{I148M}-associated NAFLD should be further looked into in detail.

5.2 Lack of TM6SF2 leads to reduced PUFA content of the membranes and altered lipid secretion (II)

5.2.1 TM6SF2 depletion increases concentrations of neutral and membrane lipids, enhances their turnover, and leads to PUFA depletion in hepatocytes

The amino acid change in TM6SF2^{E167K} leads to destabilization and degradation of the protein. Therefore we used an shRNA expressing lentivirus to generate hepatocytes in which TM6SF*2* is stably knocked down (**II, Fig. 1A**) to study the function of the protein and the effect of its variant on hepatic lipid metabolism. Consistent with earlier findings by others (Mahdessian et al. 2014, Kozlitina et al. 2014), the TAG and CE concentrations measured by ESI-MS/MS were increased in TM6SF2 knock-down hepatocytes compared with control cells treated with non-targeting shRNA lentivirus (**II, Fig. 1B-C**). Interestingly, also the concentrations of the two major membrane phospholipids PC and PE were increased in our cell model (**II, Fig. 2 C-D, inserts**). When the relative lipid species profiles of TAG, CE, PC and PE were analysed using PCA, the TM6SF2 knock-down and control cells were separated from each other in all of these classes based on the principal component 1, which clearly represented the degree of unsaturation and explained 60, 64, 88 and 93 % of the observed variation in these classes, respectively (**II, Fig. 2 A-D**). The separation of the two groups was also statistically significant in all the lipid classes except CE (SIMCA analysis, $p<0.05$). In all the classes knock-down cells were enriched in the lipid species containing SFAs and MUFAs (**II, Fig. 2**, on the right) whereas the control cells had relatively more PUFA-containing lipids (**II, Fig. 2**, on the left). In PC and PE classes, the knock-down cells contained the relatively smallest amount of the species with 20:4n-6 moieties, such as PC/PE 36:4, 38:4 and 38:5. This relative depletion of 20:4n-6 was statistically significant ($p<0.01$) in the total fatty acid profile of the cells as well (**II, Table S7**). We also determined the absolute levels of the PC species that according to ESI-MS/MS fragmentation contained 20:4n-6, and there was a reduction in their concentrations after a 24-hour culture and especially after elongated one week-long culture (**II, Fig. 3 A-B**).

In addition to the relative increase in the SFA- and MUFA-containing lipid species in the TM6SF2 knock-down cells compared to controls, there was an increase in the absolute levels of major PC species containing SFA and MUFAs in the TM6SF2 knock-down cells (**II, Fig 3. A-B**). MUFAs and especially SFAs have been shown to induce steatosis related mitochondrial dysfunction and apoptosis of hepatocytes (Malhi et al. 2006). SFAs can be converted to MUFAs by the function of SCD1, the activity of which has been found to be increased in NAFLD patients (Kotronen et al. 2009). MUFAs are major substrates for the synthesis of TAG, CE and phospholipids (Ntambi and Miyazaki 2004), and accordingly SCD1 activity has been suggested to protect the liver from lipotoxicity of SFAs in hepatic steatosis (Li et al. 2009). We studied the synthesis and turnover of lipids in our cell model by [^{13}C]glycerol and [^{3}H]acetic acid labelling. During a 24-hour [^{13}C]glycerol labelling significantly higher ($p<0.001$) amounts of labelled TAG as well as PC, PE and PI accumulated in the TM6SF2 knock-down cells compared to control cells. In addition, during a 24-hour chase period the turnover of these lipids was also higher in the TM6SF2 knock-down cells

(**II, Fig. 4 A-D**). At the end of the chase period the levels of TAG, PC and PE still remained statistically significantly higher in the TM6SF2 knock-down cells. Furthermore, [^{3}H]acetic acid labelling of the cells revealed increased incorporation of the label into TAG and CE thus confirming increased *de novo* lipogenesis of these lipids in the TM6SF2 knock-down cells (**II, Fig S1**). However, there was no difference between the two groups in glucose uptake or glycogen synthesis (**II, Fig. S2 A-B**), suggesting that the main function of TM6SF2 is related to lipid metabolism. The reasons behind the increased *de novo* lipogenesis could include increased ER stress, a phenomenon reported to exist in TM6FS2 deficiency and to induce several lipogenic activators and enzymes (Lee et al. 2012, O'Hare et al. 2017).

5.2.2 TM6SF2 depletion decreases the size of secreted lipoprotein-like particles

The finding of decreased 20:4n-6 in the TM6SF2 knock-down cells is especially interesting in the light of studies pointing to the importance of having this fatty acid in the membrane phospholipids to enable successful VLDL secretion (Rong et al. 2015, Hashidate-Yoshida et al. 2015). Importantly, mice lacking hepatic lysophosphatidylcholine acyltransferase 3 and thus having lower levels of phospholipids containing 20:4n-6 have hepatic steatosis and secrete lipid-poor VLDL deficient in 20:4n-6-containing PCs (Rong et al. 2015). In addition, patients with NASH were reported to have low hepatic PUFA levels and also specifically lower levels of 20:4n-6 in PC (Puri et al. 2007, Arendt et al. 2015). Prompted by these reports, we examined the lipoprotein-like particles secreted by the cells using electron microscopy (**II, Fig. 5A**). The size distribution of the particles showed a clear difference between the two groups; the TM6SF2 knock-down cells were almost completely lacking the largest particles (>20 nm in diameter) and secreted relatively more of the smaller particles (<15 nm) compared to the control cells (**II, Fig. 5B**). In contradiction with human and hepatic 3D spheroid data (Kim et al. 2017, Prill et al. 2019), we did not see a reduction in ApoB secretion but rather an increase in ApoB secreted by the TM6SF*2* knock-down cells (**II, Fig. 5C**), most likely reflecting the difference between a complete physiological system and an isolated cell model. However, Luukkonen *et al.* (2017), showed that humans carriers of TM6SF2^{E167K} have decreased amounts of PUFAs in liver TAGs and PCs compared to non-carriers, and that the incorporation of 20:4n-6 into TAGs and PCs of TM6SF2 knock-down hepatocytes is decreased. They hypothesized, in line with our findings, that hepatic synthesis of PUFA-containing lipids is reduced in TM6SF2^{E167K} carriers resulting in deficiency of polyunsaturated PCs in the human liver and thus impairing VLDL lipidation.

5.2.3 TM6SF2 depleted hepatocytes show impaired mitochondrial β-oxidation and have an amplified late endosomal/lysosomal compartment

In addition to impaired VLDL secretion and imbalanced lipid synthesis and turnover, the hepatic lipid accumulation associated with TM6SF2 deficiency could be due to reduced β-oxidation of fatty acids. Moreover, mitochondrial dysfunction has been reported to occur in NAFLD (Caldwell et al. 1999, Ibdah et al. 2005, Peng et al. 2018). We measured the mitochondrial β-oxidation capacity of the control and TM6SF2 knock-down cells using fatty

acid 16:0 as substrate. An experiment to measure mitochondrial stress was performed using a Seahorse® metabolic flux analyser (**II, Fig. 6A**). During an oxygen consumption rate (OCR) measurement, a basal OCR was first recorded; then, by adding oligomycin, ATP production was inhibited leaving only the proton leak to be measured. By injecting carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP), a mitochondrial uncoupling agent, an uninhibited electron flow through the electron transfer chain was enabled and maximal oxygen consumption of complex IV recorded. Finally, non-mitochondrial respiration was measured by adding rotenone and antimycin A to inhibit the respiratory chain. Based on these measurements, the basal and maximal OCRs were calculated. These results revealed that when exogenous fatty acids were utilized, the basal OCR was decreased in the TM6SF2 knock-down cells compared to control cells (**II, Fig. 6B**). However, the mechanisms that couple oxygen consumption and ATP production were not affected pointing towards TM6SF2 deficiency affecting only the processes required for β-oxidation. Increase of 20:4n-6 and 22:6n-3 in the mitochondrial membrane phospholipids has been shown to improve mitochondrial function (Khairallah et al. 2012), and it is possible that the mitochondria of TM6SF2 knock-down cells are affected by the altered cellular lipid profile and lack of PUFAs. In addition, altered ER lipid composition could disrupt the ER-mitochondrial contact sites thus reducing the flux of fatty acids into mitochondria as well as the Ca^{2+} flux between these organelles (Csordas et al. 2010).

Due to the observed membrane lipid accumulation in TM6SF2 knock-down cells, we performed electron microscopy to investigate the organelle structure of the cells. We found that the TM6SF2 knock-down cells have more late endosomes/lysosomes than the control cells (**II, Fig. 7A**), a finding that had not been reported previously. This result was confirmed using immunofluorescence microscopy and antibodies against lysosomal-associated membrane protein 1, a known lysosome marker (**II, Fig 7B**). Endosomal/lysosomal pathways are important for normal liver function and provide a means to dispose of excess lipids (Schroeder and McNiven 2014, Jaishy and Abel 2016). Thus, the noticed amplification of the late endosome/lysosome compartment may be a response to the increased lipid load of the cells.

Based on our findings, we propose the following model to explain lipid accumulation in hepatocytes due to TM6SF2 deficiency (**Figure 7**): TM6SF2 depletion leads to a decreased amount of PUFAs and especially 20:4n-6 in the ER membrane therefore disrupting lipoprotein secretion and reducing the size and lipid content of the secreted particles. This is accompanied by reduced β-oxidation of fatty acids as well as elevated membrane lipid content, increased lipid turnover and enlarged late endosome/lysosome compartment.

Since TM6SF2 deficiency leads to reduced hepatic secretion of TAG and cholesterol (Mahdessian et al. 2014, Smagris et al. 2016, Fan et al. 2016), it has been suggested that TM6SF2 could be a therapeutic target for reducing plasma lipids and the risk of cardiovascular disease (Fan et al. 2016, Li et al. 2018). Our results show that TM6SF2 depletion has many different effects on hepatocytes and on their lipid metabolism. There is also evidence on $TM6SF2^{E167}$ variant being associated with NAFLD that is more likely to progress into NASH

and hepatic fibrosis or cirrhosis (Liu et al. 2014, Dongiovanni et al. 2015), the reason for which is yet to be elucidated. For these reasons deeper understanding of the function of TM6SF2 in the liver is still required before TM6SF2 depletion could be implemented in a therapeutic setting.

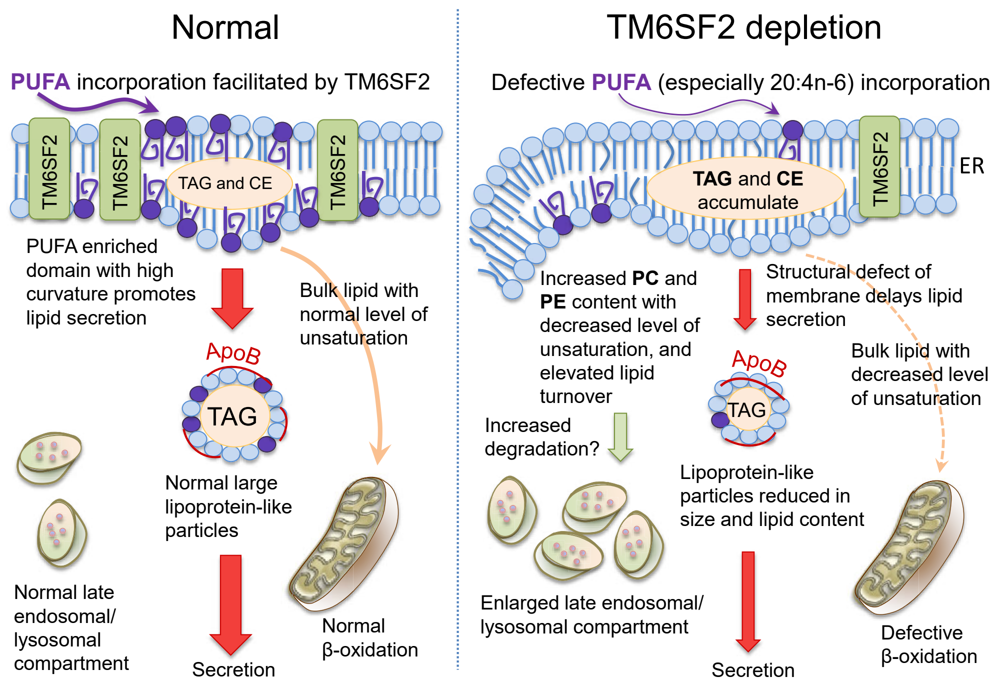

Figure 7. A model of how TM6SF2 depletion in the ER membrane leads to hepatic TAG and CE accumulation through altered lipid secretion and defective β-oxidation. Adapted from Publication II, Ruhanen *et al.* 2017 (online version).

5.3 ANGPTL3 depletion alters the lipidome of hepatocytes (III)

5.3.1 Depleting ANGPTL3 in hepatocytes alters many lipid metabolism-related pathways

The role of ANGPTL3 in circulation is well established but its intracellular function in hepatocytes has remained unknown. Nonetheless, liver-specific inhibition of the production of ANGPTL3 is a promising approach for treating cardiovascular disease (Graham et al. 2017). We knocked down ANGPTL3 in immortalized human hepatocytes (**III, Fig. 1A-C**), and performed a differential gene expression analysis followed by gene set enrichment analysis and gene set over-representation analysis to gain understanding of the pathways that ANGPTL3 depletion might affect. Both Kyoto encyclopedia of genes and genomes (KEGG) pathway analysis and Reactome overrepresentation analysis, which was performed using only statistically significantly ($p<0.05$) up/downregulated genes, highlighted several pathways

related to lipid metabolism that are altered upon ANGPTL3 depletion (**III, Table 1** and **Supplementary Figure 1**). According to these analyses, depleting ANGPTL3 changes lipid metabolism widely, affecting glycerophospholipid, sphingolipid, cholesterol and fatty acid metabolism as well as lipid signalling. Also two pathways related to longevity and three pathways related to insulin (insulin sensitivity/resistance, insulin signalling and insulin secretion) were raised by the KEGG analysis (**III, Supplementary table 3**). These are relevant findings in the light of reports showing that ANGPTL3 deficient subjects are likely to exceed the average life expectancy and have increased insulin sensitivity (Minicocci et al. 2012, Robciuc et al. 2013). In addition, it has previously been reported using the same cell model as in our studies that ANGPTL3 depletion enhances glucose uptake and down-regulates gluconeogenic genes in hepatocytes, suggesting that ANGPTL3 deficiency improves hepatic insulin sensitivity (Tikka et al. 2014).

5.3.2 ANGPTL3 depletion reduces cholesterol ester synthesis of hepatocytes

We utilized several different lipidomics approaches to study the lipid metabolism of ANGPLT3 depleted immortalized human hepatocytes (IHHs) and control cells transduced with non-targeting shRNA. There were no changes in the total levels of major membrane phospholipids PC, PE, PI or SM, analysed by ESI-MS/MS and normalised to total cellular protein. But importantly, there was a marked drop ($p<0.001$) in the total level of CEs (**III, Fig. 4A**). When the cells were labelled with [^{3}H]acetic acid or [^{3}H]oleic acid, the incorporation of both labels into CE was significantly lower ($p<0.001$) in the ANGPLT3 knock-down cells compared to control IHH cells (**III, Fig. 4E-F**), revealing that ANGPTL3 depletion reduces CE synthesis. Based on the same labelling experiments, the synthesis of unesterified cholesterol is not affected by ANGPTL3 depletion. The observed defect in CE synthesis may be explained by a reduced amount of ACAT1 in the ANGPTL3 knock-down cells, noticed both at mRNA and protein levels in our cell model (**III, Fig. 4 B-D**). The relevance of this finding in the liver *in vivo* is not clear, as ACAT2 is the major isoform needed for CE synthesis in human liver (Parini et al. 2004). In rat hepatoma cells, however, increased levels of either ACAT isoform increased CE synthesis, cellular accumulation of CEs as well as its secretion in VLDL (Liang et al. 2004), and consistently, inhibition of ACAT was shown to decrease VLDL apoB secretion in pigs (Burnett et al. 1999). ANGPLT3 LOF carriers have a reduced CE/apoB ratio of plasma VLDL and LDL when compared to non-carriers (Robciuc et al. 2013), and based on the above findings it is possible that this observation could be explained by reduced level of ACAT1.

5.3.3 ANGPTL3 deficiency causes enrichment of polyunsaturated fatty acids and depletion of monounsaturated fatty acids in hepatocytes

Even though there were no differences in the total levels of major membrane phospholipids PC, PE and PI between the control and ANGPTL3 knock-down cells, the species compositions of these lipids showed some highly interesting differences between the groups (**III, Supplementary tables 5-7**). PCA illustrated that in all these lipid classes the ANGPTL3

knock-down cells were enriched in lipid species containing PUFAs, whereas the control cells had relatively more lipid species with MUFA and SFA moieties (**III, Supplementary Figure 2A-C**). There was a similar difference between the groups also in the species composition of CE (**III, Supplementary table 8**); the relative level of the largest individual component CE18:1 was decreased (p<0.001) and the proportion of the most abundant PUFA-containing CE (CE22:6) was increased (p<0.001) in ANGPTL3 knock-down cells. Effects of ANGPTL3 depletion on hepatic phospholipid or CE profiles have not been reported before, but relative enrichment of PUFA-containing long-chain TAGs has previously been seen in livers of ANGPTL3 deficient mice (Xu et al. 2018). Unfortunately, due to the very low amounts of TAGs in IHH cells and technical limitations, we were not able to analyse the TAGs in our cell model.

The observed PUFA-enrichment and MUFA-depletion in ANGPTL3 depleted cells was evident also in the total fatty acid profile of the cells determined by gas chromatography (**III, Supplementary table 4**). The sum of MUFAs was decreased and the sums of both n-6 and n-3 PUFAs were increased in the ANGPTL3 knock-down cells compared to controls (**III, Fig. 2A**). However, the n-6/n-3 ratio was not affected by ANGPTL3 depletion. PCA of the relative fatty acid composition of the cells revealed that the fatty acids most responsible for the separation between the groups in the direction of principal component 2 were 20:5n-3 and 20:4n-6, which were enriched in the ANGPTL3 knock-down cells, and 20:3n-9, which was enriched in the control cells (**III, Fig. 2B**). Fatty acid 20:3n-9 is synthesized from the non-essential 18:1n-9, and it is an indicator of essential fatty acid deficiency (Ichi et al. 2014). According to the differential gene expression analysis, fatty acid translocase CD36 and several fatty acid binding proteins, which mediate the uptake of long-chain fatty acids and PUFAs into cells (Kane et al. 1996, Murphy et al. 2005, Ehehalt et al. 2008, Islam et al. 2014), are upregulated in the ANGPTL3 knock-down cells. This, together with the fatty acid data, suggests that fatty acid uptake may be enhanced upon ANGPTL3 depletion.

5.3.4 ANGPTL3 depletion alters the lipid mediator profile of hepatocytes

Since n-6 and n-3 PUFAs, like 20:4n-6 and 20:5n-3, which were enriched in the ANGPTL3 depleted cells, are precursors of bioactive lipid mediators (Buckley et al. 2014, Dennis and Norris 2015), we utilized an LC-MS/MS approach to study the lipid mediators produced by the ANGPTL3 knock-down and control cells (**III, Supplementary table 9**; results represent the sums of intracellular and secreted lipid mediators). Due to the limited number of samples (n=3) the differences between the groups were not statistically significant. Even so, a partial least squares discriminant analysis (PLS-DA) separated the groups from each other (**III, Fig. 3A**). Variable importance in projection (VIP) score showed the importance of each variable in separating the two groups in the PLA-DA model. All of the 15 lipid mediators with the highest VIP scores were more abundant in the ANGPTL3 knock-down cells than in the controls (**III, Fig. 3B**). There were both pro-inflammatory and pro-resolving lipid mediators among the ones with the highest VIP scores. Resolvin D6 (RvD6) had the highest VIP score of all the analysed lipid mediators. It is a specialized pro-resolving mediator (SPM), which

has been shown to induce the uptake of blood clots by macrophages, and the level of which could be raised in coronary artery disease patients by n-3 supplementation (Elajami et al. 2016). Maresin 2 (MaR2) and 22-OH-MaR1, which is a metabolite of maresin 1, are also SPMs, which have been described in macrophages and neutrophils, respectively (Deng et al. 2014, Colas et al. 2016). The SPMs 10S,17S-diHDPA and 10S,17S-diHDHA, produced from 22:5n-3 and 22:6n-3, respectively, also showed high VIP scores. The former is a protectin pathway marker (Gobbetti et al. 2017), and the latter, also known as protectin DX, has been reported to inhibit ER stress and thus attenuate hepatic steatosis in mice and insulin resistance in hepatocytes (Jung et al. 2018, Jung et al. 2019). The SPMs 13,14-dehydro,15-oxo-LXA_4 and 15-epi-LXA_4 also having high VIP scores in the data are produced from 20:4n-6 via the lipoxygenase pathway (Chandrasekharan and Sharma-Walia 2015, Pirault and Bäck 2018), and the latter of these lipid mediators is known to participate in activating the resolution phase of inflammation by down-regulating pro-inflammatory eicosanoids and by inducing the release of SPMs (Kain et al. 2017, Dakin et al. 2019). The pro-inflammatory eicosanoids, such as prostaglandins (PGs), act in the initiation phase of acute inflammation (Ricciotti and FitzGerald 2011), and prostaglandin E2 (PGE2) also plays a role in lipid mediator class switching as it initiates the resolution phase by decreasing the production of pro-inflammatory leukotriene B4 (LTB4)(Levy et al. 2001). PGD_2, PGF_{2a}, PGE_2 and LTB_4 as well as another 20:4n-6-derived pro-inflammatory lipid mediator thromboxane B2 were among the top 15 mediators with the highest VIP scores in the ANGPTL3 knock-down cells.

Lipid mediators were also analysed by grouping them based on their FA precursor. The sums of lipid mediators derived from 22:6n-3, 22:5n-3, and 20:4n-6 all showed an increasing trend in the ANGPTL3 knock-down cells (**Figure 8A**). In fact, only 20:5n-3-derived lipid mediators showed a decreasing trend in the ANGPTL3 depleted cells when compared to controls, however, resolvin E2 (RvE2) was the only species representing this group. PCA performed using sums of mass % converted values revealed that ANGPTL3 knock-down cells were relatively more enriched in 20:4n-6-derived thromboxanes and prostaglandins as well as 22:6n-3-derived protectins, maresins and resolvins (**Figure 8B**). The observed increased production of lipid mediators upon ANGPTL3 depletion is consistent with a previous report showing increased production of lipid mediators after PUFA (20:4n-6, 20:5n-3 and 22:6n-3) supplementation and enrichment in membrane phospholipids (Holopainen et al. 2019). In our cell model, two isoforms of cytosolic PLA2 were upregulated based on the differential gene expression analysis. This enzyme, which releases fatty acids from glycerophospholipids, is reported to show substrate specificity for 20-22-carbon PUFAs (Shikano et al. 1994, Batchu et al. 2016). Thus, increased substrate availability likely potentiates lipid mediator production in the ANGPTL3 depleted cells. In addition, it has been shown that when cells have increased amounts of bioactive PUFAs, these fatty acids can be elongated to a less active form (Akiba et al. 2000, Zou et al. 2012, Dong et al. 2016, Tigistu-Sahle et al. 2017, Holopainen et al. 2019). Fatty acid 20:4n-6 can be elongated to adrenic acid 22:4n-6, which is a less potent activator of cyclooxygenase, the key enzyme needed in prostaglandin biosynthesis (Zou et al. 2012, Dong et al. 2016). Similarly, 20:5n-3 can be elongated to 22:5n-3 (Akiba et al. 2000), which, however, is also a precursor for pro-resolving lipid mediators (Dalli et al. 2013, Dalli et al. 2015). The relative amount of both 22:4n-6 and 22:5n-3 was significantly increased in

the ANGPTL3 knock-down cells when compared to controls, possibly reflecting a response evoked in order to attenuate the synthesis of 20:4n-6 and 20:5:n-3 -derived lipid mediators.

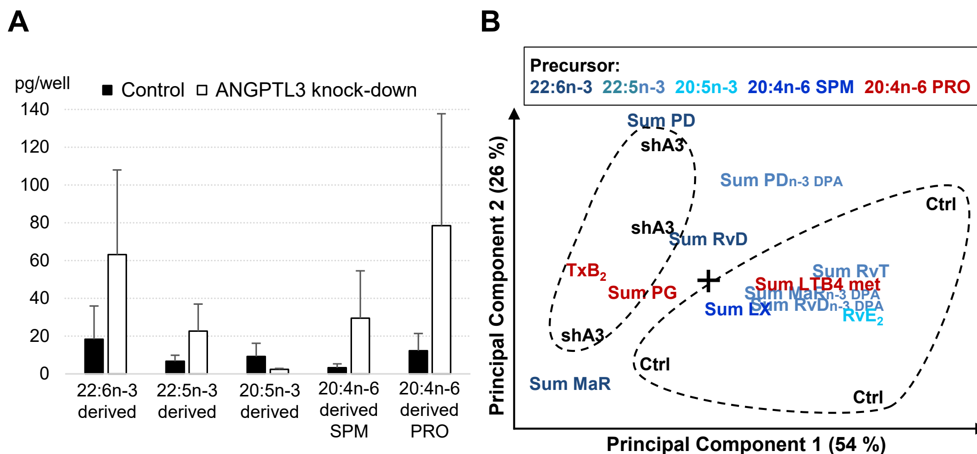

Figure 8. Knocking down ANGPTL3 alters the profile of synthesized lipid mediators. (A) Sums of lipid mediators grouped by their precursor fatty acids. The values represent mean ± SEM, n=3. (B) PCA of the sums of different lipid mediator groups (mass%). Ctrl=control cells treated with non-targeting RNA, ShA3=cells treated with shRNA against ANGTPL3, SPM=specialized pro-resolving mediator, PRO=pro-inflammatory mediator, MaR=maresin, TxB_2=thromboxane B2, PD=protectin, PG=prostaglandin, RvE=E series resolvin, $RvD_{n\text{-}3DPA}$=D series resolvin derived from 22:5n-3, LX=lipoxin, LTB4met=leukotriene B4 metabolites.

5.4 Lack of ANGPTL3 leads to changes in the core and surface lipids of lipoproteins (III)

5.4.1 ANGPTL3 deficiency changes the fatty acid profile of lipoproteins

To gain understanding on how lipoproteins secreted by the liver are affected by ANGPTL3 deficiency, we analysed VLDL, LDL and HDL lipoprotein fractions derived from plasma of subjects who were either homozygous for an ANGPTL3 LOF variant or did not carry the variant at all. The subjects selected for this study present a subpopulation of a larger study cohort (Minicocci et al. 2016, Tikkanen et al. 2019), and there is no difference between the two groups in dietary intake, physical activity, smoking prevalence, or use of anti-inflammatory medications. Fatty acid profiles of the lipoproteins were determined by gas chromatography (**III, Supplementary table 10**). When examining molar percentages of individual fatty acids, the most pronounced difference between the groups was the elevated amount of 18:2n-6 in the ANGPTL3 deficient subjects. This change was most prominent in the VLDL fraction ($p<0.001$) but remained statistically significant also in LDL ($p<0.01$) and HDL ($p<0.05$) fractions. The molar percentage values were then standardised in order to evaluate also the effect of the smaller components in the data and a PCA was performed using all the lipoprotein fractions in the same analysis. A PCA biplot separated the groups from each other, principal component 1 according to the lipoprotein fraction (VLDL on the right,

LDL in the middle, and HDL on the left) and principal component 2 according to the sample group (ANGPTL3 LOF homozygotes higher up and control subjects in the bottom) (**III, Fig. 5**). The fatty acids on the right and enriched in the VLDL fractions were mainly saturated or monounsaturated and 14-18 carbon-long, whereas the fatty acids enriched in the HDL fractions had 20 carbons or more and many of them were also polyunsaturated. In addition, plasmalogen-derived dimethyl acetals were enriched in the HDL fraction of the control subjects. LPL hydrolyses lipoproteins in the circulation, and its hydrolysis efficiency decreases with increasing chain length and unsaturation (Wang et al. 1993, Sato et al. 1999). In addition to hydrolysing TAG, LPL has PLA1 activity (McLeanBest et al. 1986), but it hydrolyses only ester bonds and not ether bonds (McLeanDemel et al. 1986, Olivecrona and Bengtsson-Olivecrona 1987, Griffon et al. 2006). Thus the function of LPL would seem to explain the change in fatty acid quality in the direction of principal component 1. Principal component 2 separated the ANGPTL3 LOF carriers and control subjects, and thus reflected the presence/absence of ANGPTL3 in the circulation of the subjects. Accordingly, ANGPTL3 deficient subjects have increased LPL activity (Robciuc et al. 2013), but there is no significant difference in the activities of EL, CETP or PLTP between the ANGPTL3 LOF carriers and control subjects (Robciuc et al. 2013, Minicocci et al. 2016).

5.4.2 ANGPTL3 deficiency changes the quality of surface and core lipids of lipoproteins

We determined the detailed profile of the core and surface lipids of the lipoprotein fractions by ESI-MS/MS. The total amounts of lipids at the class level were lower in the lipoprotein particles of the ANGPTL3 LOF carriers when compared to controls (**III, Supplementary table 11**). This finding is consistent with previous reports and highly relevant in terms of the reduced cardiovascular risk mediated by ANGPTL3 deficiency (Musunuru et al. 2010, Robciuc et al. 2013, Stitziel et al. 2017). At the lipid species level ANGPLT3 deficiency lead to compositional changes in all the lipoprotein classes analysed (**III, Supplementary tables 12-16**), and the changes in TAG, PC and lysoPC species likely reflect the increased LPL activity observed in the plasma of these subjects (Robciuc et al. 2013). TAGs and lysoPCs of the ANGLPTL3 LOF carriers were enriched in PUFA-containing species (**III, Fig. 6, 7C; Supplementary tables 12, 15**). LPL hydrolyses the fatty acids with the least number of carbons and double-bonds most efficiently, leaving the long-chain PUFAs to be hydrolysed last (Wang et al. 1993, Sato et al. 1999). Moreover, the PLA1 activity of the enzyme depends on the structure of the fatty acid in the *sn*-2 position. The longer the fatty acid in the *sn*-2 position the more efficiently LPL hydrolyses the ester bond in the *sn*-1 position of the phospholipid (McLean and Best et al. 1986). In PCs of all the lipoprotein fractions of the ANGPTL3 LOF carriers, there was a clear enrichment of alkyl-ether species (**III, Supplementary table 16),** which could be explained by the ester bond-specificity of LPL (McLean and Demel et al. 1986, Olivecrona and Bengtsson-Olivecrona 1987, Griffon et al. 2006). Another plausible explanation could be related to the function of peroxisomes as ether lipids are synthesized in these organelles (van den Bosch et al. 1993). However, there are no reports on altered peroxisomal function of ANGPTL3 deficent subjects.

CEs of the lipoproteins derived from ANGPTL3 LOF carriers had an elevated level of species with 16:1 and 18:1 fatty acid moieties when compared to control subjects (**III, Supplementary table 13**). CEs are not hydrolysed by LPL since the enzyme is specific for ester bonds on the glycerol backbone (McLean and Demel et al. 1986, Olivecrona and Bengtsson-Olivecrona 1987, Griffon et al. 2006). The observed compositional change could be related to the function of LCAT as it transfers fatty acids from the *sn*-2 potion of PC to cholesterol in lipoproteins (Glomset 1962, Chen and Albers 1982), but the mechanism is poorly studied and would require further investigation. The compositional differences seen in the lipids of circulating lipoproteins of ANGPTL3 LOF carriers and control subjects could also, at least in part, originate from the lipid composition of the liver. At the moment, there are no reports on liver lipid composition of ANGPTL3 deficient subjects, so this question remains open.

Also the composition of SM and its ratio to PC were altered in the ANGPTL3 LOF carriers. The SM/PC ratio of lipoproteins derived from the ANGPTL3 LOF carriers was statistically significantly increased when compared to control subjects (**III, Fig. 7A**). PLTP transfers SM efficiently (Huuskonen et al. 1996), and thus it is possible that the increased SM/PC ratio is present already in nascent VLDL particles, which are then hydrolysed by LPL in the circulation, after which PLTP could transfer the extra surface lipids to HDL and further to LDL (Albers et al. 2012). An increased SM/PC ratio has been shown to increase the capacity of HDL to collect cholesterol from cells (Horter et al. 2002). However, SM enrichment in HDL also inhibits esterification of cholesterol by LCAT (Subbaiah and Liu 1993), and thus the effect of increased SM/PC ratio on reverse cholesterol transport is not clear. The proportion of long-chain SMs 24:1 and 24:2 was increased and the relative amount of short saturated SMs was decreased in the lipoproteins of ANGPTL3 LOF carriers when compared to control subjects (**III, Fig. 7B**). It has been reported that saturated SM species as well as SM 16:1 increase LDL aggregation and the risk of cardiovascular death (Ruuth et al. 2018). The lipoproteins of ANGPTL3 LOF carriers could thus be less prone to aggregate even though SM is enriched in the surface of these particles. This could provide further protection against cardiovascular disease on top of the low levels of CEs and TAGs in circulating lipoproteins of the ANGPTL3 deficient subjects.

6 CONCLUSIONS AND FUTURE PERSPECTIVES

The work presented in this thesis provides new knowledge of the function of proteins PNPLA3 and TM6SF2 as well as of the mechanisms how their variants affect hepatic lipid accumulation. In addition, detailed analysis of the effects of ANGPTL3 depletion on the lipidome of hepatocytes and secreted lipoprotein particles is reported for the first time. The main findings of the thesis are summarised in **Figure 9**. In the first study of this thesis, we demonstrated that PNPLA3 is a TAG remodelling protein and that PNPLA3^{I148M} is a LOF variant that is also more extensively associated with lipid droplets. Hepatic TAG accumulation could be related to the ineffective remodelling of TAGs by PNPLA3^{I148M}, or accumulation of PNPLA3^{I148M} on lipid droplets may lead to more hydrolysis-resistant lipid droplets thus causing hepatic lipid accumulation (BasuRay et al. 2017, Negoita et al. 2019, Wang et al. 2019). In the second study, we mimicked the effect of TM6SF2^{E167K} by knocking down TM6SF2 in hepatocytes and showed that TM6SF2 depletion changes the lipid composition of membranes by reducing the amount of PUFAs and increasing the levels of SFAs and MUFAs. The lack of PUFAs in membranes leads to secretion of smaller lipoprotein-like particles and accumulation of TAG and CE in the cells. We also observed reduced β-oxidation in the TM6SF2 depleted cells, which can additionally lead to hepatic lipid accumulation. In the third part of this thesis, we showed that ANGPLT3 depletion has extensive effects on the lipid metabolism of hepatocytes. PUFAs were enriched in lipids of ANGPTL3 depleted cells, coinciding with enhanced lipid mediator production. In addition, cholesterol ester synthesis was reduced in ANGPTL3 knock-down hepatocytes. The changes in core and surface lipids of lipoproteins caused by ANGPTL3 deficiency most likely reflected the increased activity of LPL, the activity of which decreases with increasing chain length and unsaturation (Wang et al. 1993, Sato et al. 1999).

Many of the treatment options currently available for NAFLD are targeting obesity and related metabolic disorders (European Association for the Study of the Liver (EASL) et al. 2016, Romero-Gomez et al. 2017, Ganguli et al. 2019). Since obesity further increases the risk of developing NAFLD due to the PNPLA3^{I148M} and TM6SF2^{E167K} variants, the carriers of these risk alleles are expected to benefit most from weight-loss interventions (Stender et al. 2017, Wang et al. 2018). However, understanding the mechanisms of the hepatic lipid accumulation in NAFLD associated with these genetic variants may provide further treatment options and improve therapeutic approaches. For example, n-3 supplementation, which is currently used in treating NAFLD, can be harmful in terms of hepatic lipid accumulation in patients homozygous for PNPLA3^{I148M} (Scorletti et al. 2015).

In line with our findings of the effects of TM6SF2 depletion, decreased amounts of PUFAs have been observed in liver TAGs and PCs of human subjects carrying the TM6SF2 ^{E167K} allele, and the incorporation of 20:4n-6 into TAGs and PCs has been shown to be decreased in TM6SF2 knock-down hepatocytes (Luukkonen et al. 2017). Thus in the case on TM6SF2, different strategies using fatty acid supplementations or other manipulations to increase the levels of PUFAs in the livers of these subjects could provide means for prevention and

treatment of TM6SF2 ^{E167K}-associated NAFLD. Due to the plasma lipid lowering effects of TM6SF2 depletion and TM6SF2 ^{E167K} variant, TM6SF2 reduction has been proposed to be a possible strategy for treating cardiovascular disease (Fan et al. 2016, Li et al. 2018). However, the mechanisms of hepatic lipid accumulation related to TM6SF2 deficiency should be understood in more detail before considering such approaches.

There is accumulating evidence on the roles of PUFA-derived lipid mediators in the resolution of inflammation in atherosclerosis (Akagi et al. 2015, Fredman et al. 2016, Gerlach et al. 2019, Bäck et al. 2019). We saw an elevation of PUFA-derived lipid mediators in the ANGPTL3 depleted hepatocytes, and many of these lipid mediators have roles in resolution of inflammation, recovery from cardiovascular events, and also in attenuating hepatic steatosis and insulin resistance (Kain et al. 2017, Jung et al. 2018, Jung et al. 2019, Dakin et al. 2019). ANGPTL3 deficient subjects have increased insulin sensitivity (Robciuc et al. 2013), and liver-specific mechanisms have been suggested to be involved this phenotype (Tikka et al. 2014). Interestingly, NASH patients have been reported to have elevated levels of circulating ANGPTL3 (Yilmaz et al. 2009), and ANGPTL3 deficient subjects are not known to have increased liver fat or suffer from other adverse clinical outcomes (Minicocci et al. 2012). Thus, ANGPTL3 deficiency would seem to result in favourable outcomes in the liver. However, further studies on hepatic depletion of ANGPTL3 are needed. In the future, an analysis of the lipid mediators derived from the plasma of ANGPTL3 deficient subjects could provide further clues of the mechanisms behind the cardioprotective effects of ANGPTL3 deficiency beyond the decreased levels of plasma lipids.

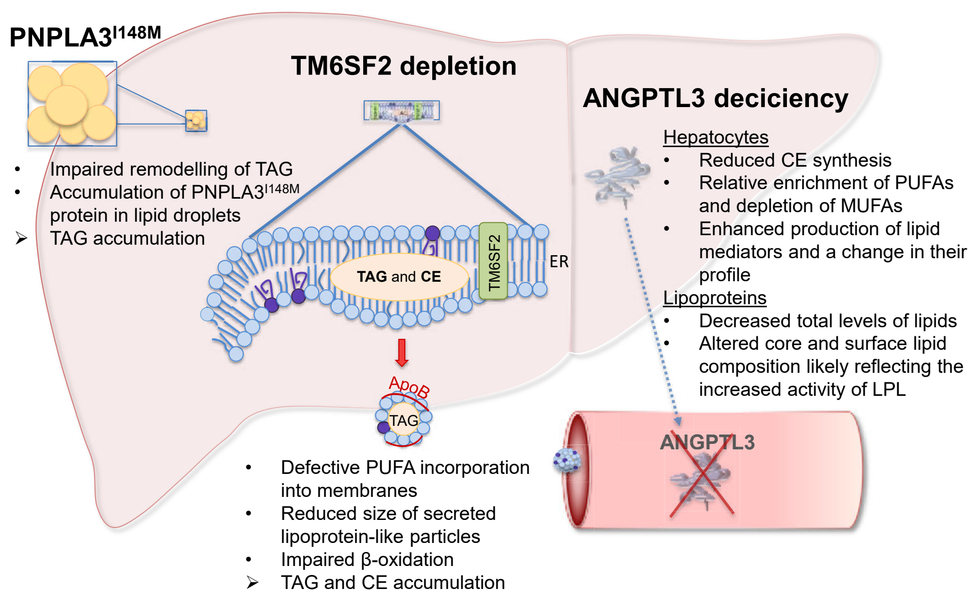

Figure 9. The effects of PNPLA3^{I148M}, TM6SF2 depletion and ANGPTL3 deficiency. A summary of the findings of publications I-III.

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