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IDENTIFICATION AND FUNCTIONAL CHARACTERIZATION OF PROTEINS INVOLVED IN HEPATIC TRIGLYCERIDE METABOLISM

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Identification and functional characterization of proteins involved in hepatic triglyceride metabolism
THESIS FOR DOCTORAL DEGREE (Ph.D.)

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To my beloved family, whomever this may include

ABSTRACT

Triglycerides are the main form of energy in the tissues and liver, along with the adipose tissue, is the main organ of triglyceride metabolism and storage in the lipid-droplet organelles. A number of proteins are involved in the regulation of the triglyceride metabolism in human liver, however their specific role is still not thoroughly known. The aim of this thesis is to evaluate the functional role of three proteins in triglyceride regulation in an experimental model of human liver.

In Paper I we identified the gene Transmembrane 6 superfamily member 2 (*TM6SF2*) as the putative cause for the association between the *19p12* locus with plasma triglyceride levels and non-alcoholic fatty liver disease, by employing expression studies and expression quantitative trait locus analysis in 206 human liver samples. *TM6SF2* encodes a protein of 351 amino acids localized in the Endoplasmic reticulum (ER) and the ER-Golgi intermediate compartment, as investigated in human hepatoma cells. Functional studies showed that *TM6SF2* siRNA inhibition led to reduced secretion of triglyceride-rich lipoproteins (TRLs) and increased cellular triglyceride concentration and number of lipid-droplets, however the putative pathophysiological mechanism of these observations is still unclear.

In Paper II we investigated the physiological functions of Patatin-like phospholipase domain containing proteins 2, 3 and 4 (*PNPLA2, PNPLA3* and *PNPLA4*), as potential triglyceride hydrolases in Huh7 and HepG2 human hepatomas. We found that siRNA inhibition of *PNPLA3* or *PNPLA4* is not associated with changes in triglyceride hydrolysis, TRL secretion or cellular triglyceride accumulation. However, *PNPLA2* siRNA inhibition reduced intracellular triglyceride hydrolysis and decreased TRL secretion, both in the absence or presence of oleate-containing medium or of the PNPLA2 inhibitor Atglistatin. In contrast, we found no effects of *PNPLA2* inhibition on lipid-droplet homeostasis. Visualization analysis with confocal microscopy found significant co-localization of PNPLA2 with the ER, but no clear evidence for PNPLA2 localization around the lipid-droplets. This data indicates that PNPLA2 hydrolyses a triglyceride compartment comprising of very small lipid-droplets that are involved in the regulation of TRL secretion, but are not detectable by confocal microscopy.

In Paper III we studied the likely role of Abhydrolase domain-containing 5 (ABHD5) as the co-activator of PNPLA2 in the regulation of hepatic triglyceride metabolism. We employed siRNA inhibition techniques in Huh7 hepatoma cells and showed that *ABHD5* siRNA inhibition reduced triglyceride hydrolysis and decreased TRL secretion while there was no effect on cellular triglyceride content. These results are similar to the effects of *PNPLA2* siRNA inhibition on triglyceride metabolism as examined in Paper II. We also found no additive effects of combined *ABHD5-PNPLA2* siRNA inhibition in hepatic triglyceride metabolism. We employed confocal microscopy analysis and observed localization of ABHD5 in the ER, but not in Golgi or around the lipid-droplets, while a significant co-localization of ABHD5 and PNPLA2 was observed. These observations suggest that ABHD5

is a co-activator of PNPLA2 with no separate triglyceride hydrolysis activity in human hepatocytes.

Overall, this Thesis identifies TM6SF2 as a membrane protein regulating the TRL secretion in Huh7 and HepG2 hepatoma cells. It also demonstrates the role of triglyceride hydrolysis in the regulation of TRL secretion where PNPLA2 is the main triglyceride hydrolase activated by ABHD5. Finally, it suggests the existence of very small lipid-droplets containing the substrate compartment of the PNPLA2- and ABHD5-mediated triglyceride hydrolysis.

LIST OF SCIENTIFIC PAPERS

I. Mahdessian H, Taxiarchis A, Popov S, Silveira A, Franco-Cereceda A, Hamsten A, Eriksson P, Hooft FV. TM6SF2 is a regulator of liver fat metabolism influencing triglyceride secretion and hepatic lipid droplet content. Proceedings of the National Academy of Sciences. 2014;111(24):8913-8918. doi:10.1073/pnas.1323785111.

II. Taxiarchis A, Mahdessian H, Silveira A, Fisher RM, Hooft FMVT. PNPLA2 influences secretion of triglyceride-rich lipoproteins by human hepatoma cells. Journal of Lipid Research. 2019;60(6):1069-1077. doi:10.1194/jlr.m090928.

III. Taxiarchis A, Silveira A, Fisher RM, Hooft FMVT. The PNPLA2 co-activator ABHD5/CGI-58 influences the secretion of triglyceride-rich lipoproteins by human hepatoma cells. *Manuscript*

CONTENTS

LIST OF ABBREVIATIONS

ABHD5	Alpha-beta hydrolase domain-containing 5
APOB	Apolipoprotein B
CHD	Coronary heart disease
CVD	Cardiovascular disease
DGAT	Diacylglycerol O-acyltransferase
ER	Endoplasmic reticulum
FA	Fatty acid
GWA	Genome-wide association
HDL	High density lipoprotein
LD	Lipid Droplet
LDL	Low-density lipoprotein
MTTP	Microsomal triglyceride transfer protein
NAFLD	Non-alcoholic fatty liver disease
NEFA	Non-esterified fatty acids
PDI	Protein disulfide isomerase
PLIN2	Perilipin2
PNPLA2	Patatin-like phospholipase domain-containing protein 2
siRNA	Small interfering RNA
TG-TAG	Triglyceride
TM6SF2	Transmembrane 6 superfamily 2
TRL	Triglyceride-rich lipoprotein
VLDL	Very low density lipoprotein

1 INTRODUCTION

1.1 TRIGLYCERIDES AND LIVER

Triglyceride is the most concentrated energy form found in biological tissues [1]. It is an ester consisting of three fatty acids and one glycerol backbone. Fatty acids are the essential substrates for membrane lipids and lipids involved in cellular signaling. However, high concentrations of non-esterified fatty acids (NEFA) distort the integrity of cellular membranes, alter the intracellular pH balance and evoke the production of harmful bioactive lipids. This leads to lipotoxicity, a condition characterized by cellular stress, organelle dysfunction and cell death. In contrast, triglycerides show low biological toxicity and are well tolerated in the tissues and cells. Thus, they provide a "safe" and efficient form of fatty acid storage and transportation via the process of triglyceride synthesis and triglyceride hydrolysis. Triglycerides are thus part of a well-regulated mechanism ensuring the esterification of fatty acids to triglycerides and cholesterol esters and providing a balance between the fatty-acid uptake, storage, utilization and secretion in the tissues [2].

Triglycerides are synthesized in most tissues by the acyl CoA:diacylglycerol acyltransferase (DGAT) 1 and 2 enzymes, as reviewed in [3], however they are stored only in few tissues; skeletal muscle and the heart can store tiny amounts of triglycerides while the adipose tissue and the liver are the most important storage pools of neutral lipids, storing them in specialized intracellular organelles called lipid-droplets [4]. However, other cells also store lipids in lipid-droplets, including enterocytes, macrophages and adrenocortical cells.

The liver is the central organ responsible for lipid homeostasis. Hepatocytes are the major cell-type responsible for the triglyceride metabolism and storage in the lipid droplets. Additional cell types within the liver include stellate cells, Kupffer cells, biliary epithelial cells and liver sinusoidal endothelial cells, all of which have specialized and unique physiological functions [5].

1.2 INTRACELLULAR LIPID METABOLISM

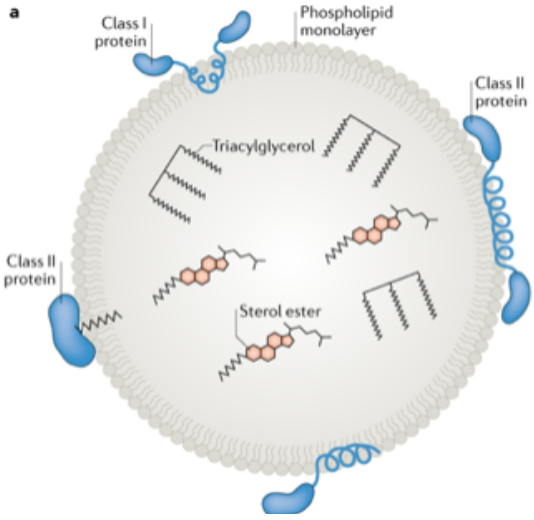

Figure 1. Schematic representation of a lipid-droplet illustrating their complexity and overall structure. Reproduced with permission from Olzmann & Carvalho [11].

In a number of excellent reviews, lipid-droplets are described as a necessary cell compartment of lipid reservoirs for cell membrane components and metabolic energy [6–10]. Lipid-droplets are mostly formed in the ER and they have a nonpolar, neutral lipid core comprising of triglycerides, cholesterol esters and/or retinol esters (*Figure 1*) [11]. The cytosolic triglyceride pools derive from esterification of the following sources: 1. fatty acids originating from *de novo* lipogenesis, 2. exogenous-derived NEFA circulating in plasma and 3. fatty acids deriving from the uptake and hydrolysis of lipoprotein remnants [12]. The lipid-droplet core is surrounded by a phospholipid surface monolayer of phosphatidylcholine with lesser amounts of phosphatidylethanolamine, phosphatidylinositol, lyso-phosphatidylcholine, and lyso-phosphatidylethanolamine.[13] There are two main classes of lipid-droplet-associated proteins: proteins stably associated with membranes that partition between lipid-droplets and the ER (class I proteins) and proteins recruited directly from the cytosol to the lipid droplet surface (class II proteins) [11]. Depletion of proteins that change the phospholipid content dramatically changes lipid-droplet morphology [14–17]. Some proteins bind to the lipid-droplet surfaces and regulate their size and number. These proteins include, among others, the Perilipin2 (PLIN2) protein which is highly expressed in liver [18,19].

Imbalances in the regulatory mechanisms of lipid homeostasis in human liver may lead to steatosis, the unphysiologically high concentration of triglyceride in the liver cells (usually defined as hepatic triglyceride content > 5%). These imbalances include increased substrate availability for triglyceride synthesis and lipid-droplet formation as well as impaired triglyceride mobilization due to disturbances in lipid-droplet hydrolysis, fatty-acid oxidation or TRL assembly and secretion [20].

Steatosis is considered as the first stage of nonalcoholic fatty liver disease (NAFLD), a spectrum of liver disease in the absence of high alcohol consumption. The disease spectrum comprises of, initially, hepatic steatosis, developing through hepatic steatohepatitis (presence of liver fat in combination with liver inflammation and degeneration), resulting to fibrosis and ultimately to cirrhosis or in some cases to hepatocellular carcinoma [21]. The prevalence of nonalcoholic fatty liver disease is increasing worldwide representing a serious public health problem and is strongly associated with type 2 diabetes mellitus and cardiovascular

disease. Weight loss is currently the only effective treatment option for NAFLD with no pharmaceutical treatments yet approved [21,22].

The main hallmark feature of hepatic steatosis is the triglyceride accumulation in the lipid-droplets and appears in two morphological variations: in macrovesicular steatosis large lipid droplets are present in the cytoplasm and displace the nucleus whereas in microvesicular steatosis a high number of smaller lipid-droplets accumulates within the hepatic cytoplasm with the nucleus remaining intact [23]. Overall, it becomes clear that impairments in the lipid-droplet homeostasis including biogenesis and triglyceride mobilization may lead to hepatic steatosis and cardiovascular disease.

1.2.1 Biogenesis of lipid-droplets

The mechanism of lipid-droplet biogenesis is still poorly understood despite the recent discoveries and it comprises of a number of different steps, as shown in *Figure* 2 and reviewed in [11,24].

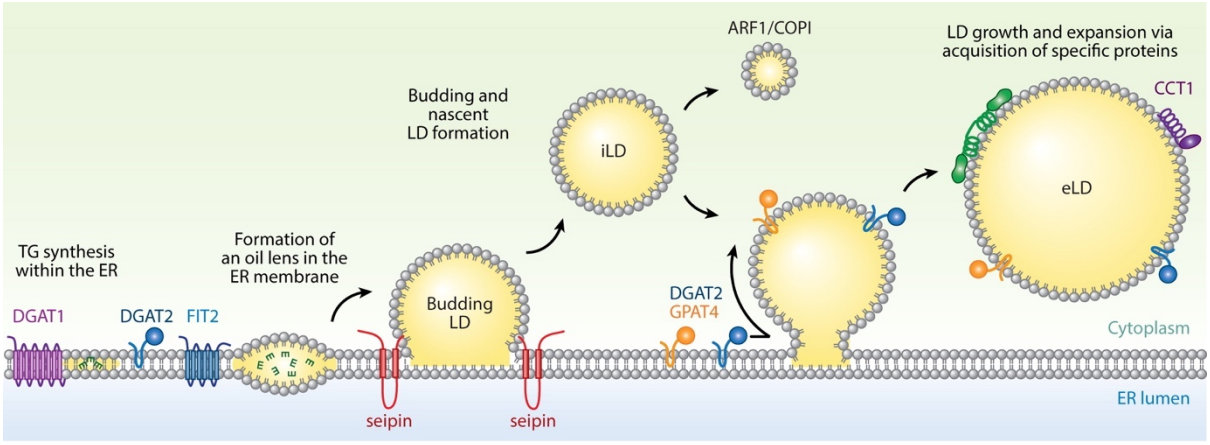

Figure 2. Schematic representation of lipid droplet biogenesis. Reproduced with permission from Walther TC et al [24].

Step 1. Triglyceride synthesis within the ER. This is a result of esterification of activated fatty acids into triglycerides and cholesterol esters. Triglycerides are synthesized mainly by the diacylglycerol acyltransferases (DGAT1 and DGAT2). These enzymes are localized primarily in the ER. It has been suggested that, despite the fact that DGAT1 and DGAT2 can compensate for each other for triglyceride synthesis, these triglyceride synthesis enzymes have different roles at least in primary hepatocytes; the triglyceride which is synthesized by DGAT1 is preferentially channeled to oxidation, whereas DGAT2 synthesizes triglyceride destined for very low-density lipoprotein assembly [25]. The synthesized triglyceride accumulates in an initial lens-like shape within the ER membrane bilayer.

Step 2. Formation of an oil lens. Neutral lipid deposition occurs between the two leaflets of the ER bilayer [26]. Once the concentration of neutral lipids exceeds a critical threshold, lipid (oil) lenses begin to shape and grow. No proteins have been identified to play a direct role in lens formation.

Step 3. Budding of lipid-droplets. At some point, the growing structure is predicted to bud off the ER, forming an "initial" lipid-droplet (iLD). According to this model, the lipid-droplet budding occurs spontaneously when sufficient triglycerol has influxed, in combination with lower tension and elastic moduli on the lipid-droplet surface [27]. Seipin, an ER membrane protein, has been shown to play a major role in correct lipid-droplet budding by allowing more triglycerides to be added into the newly formed lipid-droplets[15] [28]. After budding, lipid-droplets grow and expand. This is achieved in two different ways; first, by fusion of one or more nascent lipid-droplets via ER membrane bridges, which allows the transfer of triglyceride and formation of one bigger lipid-droplet. Second, through re-localization of several enzymes from the ER to the lipid-droplet surface which enables triglyceride synthesis directly on the lipid-droplet surface, thus making the newly formed triglyceride to accumulate in the lipid-droplet core [29].

1.2.2 Lipid-droplet hydrolysis

Fatty acids are mobilized from the lipid-droplet core with the purpose of providing the cell with substrate for its metabolic needs. This is mainly done with lipolysis (triglyceride hydrolysis) which has been extensively studied and clarified in the human adipose tissue (*Figure 3*). In adipose tissue, lipid-droplet hydrolysis generates fatty acids for secretion by adipocytes. In the liver, lipid-droplet hydrolysis provides fatty acids for β-oxidation, cellular signaling, phospholipid precursors for cell membranes as well as the necessary substrates for the assembly and secretion of lipoproteins [30].

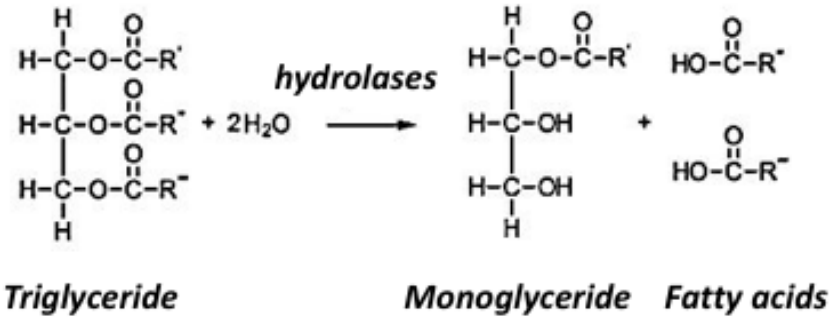

Figure 3. Overview of triglyceride hydrolysis

The first step in the triglyceride hydrolysis in adipocytes is catalyzed by PNPLA2, also known as Adipose triglyceride lipase (ATGL) which was identified as a protein-encoding gene with abundant triglyceride lipase activity [31] and established as the triglyceride lipase in murine and human adipose tissue [32]. PNPLA2 encodes a 504–amino acid protein with 86% identity to the mouse enzyme. *PNPLA2* is also expressed in the cardiac muscle and liver, however at lower levels compared to the adipose tissue. Previous studies have shown that knockdown or inactivation of *PNPLA2* in murine liver leads to hepatic steatosis in mice and decreased triglyceride hydrolysis activity in primary murine hepatocyte cultures whereas overexpression in mouse liver ameliorates hepatic steatosis. This data provides support for a role of *PNPLA*2 in hepatic triglyceride hydrolysis and metabolism in rodents, however there are no studies elucidating the role of PNPLA2 in human hepatocytes [30]. *PNPLA3* and *PNPLA4,* two additional members of the PNPLA2 gene-family expressed in human hepatocytes, have been shown to have *in vitro* triglyceride hydrolase activity. However, the functional significance of *PNPLA3* and *PNPLA4* in human liver triglyceride metabolism has not been established, as reviewed in [33].

The next steps of triglyceride lipolysis involve the cleavage of diglycerol into monoglycerol and the subsequent cleavage of monoglycerol to glycerol and fatty acids. These processes are catalyzed in adipose tissue by hormone-sensitive lipase and monoglyceride lipase respectively, as reviewed in [34] . These enzymes have been well-characterized in the adipose tissue but it has been suggested that other triglyceride hydrolases are involved in the human liver as well.

In addition to the previous enzymes, the process of triglyceride hydrolysis is controlled by a number of proteins and co-factors. Abhydrolase domain containing-5 protein (ABHD5), also known as CGI-58, is a member of an esterase/thioesterase/lipase gene family. It has been implicated in having a role in triglyceride hydrolysis, as well as in TRL assembly and secretion, via activation of PNPLA2 as investigated in adipocytes and myocytes [35,36]. Liver-specific inhibition of ABHD5 in mice has shown to induce hepatic steatosis [37,38] and reduction in triglyceride hydrolase activity and TRL secretion [37]. Similarly, rat hepatoma cells deficient in ABHD5 exhibit accumulation of cellular triglyceride in combination with a decrease in triglyceride and APOB-VLDL secretion due to decreased triglyceride hydrolysis [39,40]. Despite the role as a co-activator of PNPLA2, it has been recently proposed that ABHD5 may exhibit lipolytic activities at a PNPLA2-independent manner [41]. Nevertheless, little is known about the function of ABHD5 in human liver and whether it possesses lipolytic activities independently of PNPLA2.

1.2.3 TRL assembly and secretion

The assembly and secretion of TRLs from the liver has been analyzed for more than 50 years and the results of these studies have been summarized in a number of excellent review articles [42–46]. An important breakthrough was the identification of two APOB proteins, APOB100 and APOB48, the essential structural components of TRLs secreted by the liver and intestine, respectively [47]. The subsequent cloning of APOB provided the tools to study in more detail the biosynthesis and intracellular transport of APOB in hepatocytes using, for example, pulse-chase methods in different liver-cell models. These studies demonstrated that the hepatic TRL assembly process starts with the lipidation of the growing APOB100 protein in the lumen of the ER. This lipidation process continues until the precursor TRL has acquired sufficient quantities of triglycerides to permit APOB to fold correctly on the particle. Further additions of triglyceride occur in the transition of the lipoprotein particle from the ER to the Golgi. The mature TRL is subsequently secreted by the hepatocyte via an as yet ill-defined transport process.

There is evidence that APOB100 is constitutionally expressed in the liver, indicating that transcriptional regulation of APOB is of minor importance in the regulation of TRL secretion. It was found that the availability of lipids, in particular triglycerides, is a crucial factor in the regulation of TRL assembly and secretion [48]. It was shown that the lack of triglycerides leads to the retention of precursor TRLs in the secretory pathway and ultimately induces a proteosomal degradation process of precursor TRLs. It was therefore proposed that post-transcriptional degradation of APOB constitutes an important mechanism for the regulation of TRL synthesis and secretion. Surprisingly, the regulation of the supply of triglycerides for TRL synthesis has not been studied in detail and little is known regarding the involvement of specific proteins/enzymes in this process.

1.3 LIPOPROTEIN METABOLISM

Triglycerides and cholesterol esters are crucial sources of energy and building-blocks of membranes. Since these molecules are insoluble in water, they are transported to different organs through the circulation in association with apolipoproteins, forming particles called "lipoproteins". Thus, the lipoproteins play a pivotal role in the transport of dietary lipids from the intestine and the liver to the peripheral tissues as well as the reverse cholesterol transport.

1.3.1 Classes of lipoproteins

There are different types of lipoproteins but all of them consist of specific apolipoproteins, nonesterified cholesterol and phospholipids spherically surrounding a neutral-lipid core that contains triglycerides and cholesterol esters. The surface apolipoproteins can be APOA-I, APOA-II, APOA-IV, APOA-V, APOB48, APOB100, APOC1, APOCII, APOE and APO(a). The plasma lipoproteins are divided into seven classes which are shown in *Figure 4* [49,50].

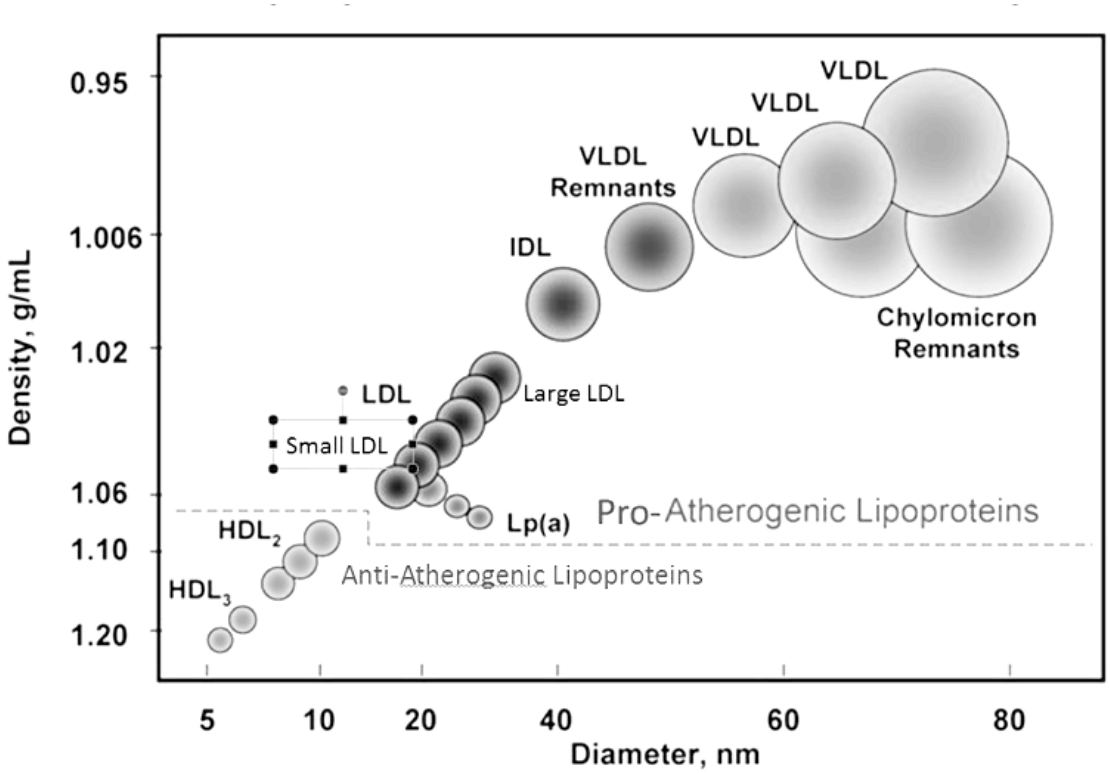

Figure 4. Classes of lipoproteins. Reproduced with permission from Feingold & Grunfeld [49].

In summary, the lipoprotein division is dependent upon their size (density), lipid composition and type of surface apolipoprotein. The different lipoproteins, given by their name and their size from the smallest to the largest scale, are the following [51]; chylomicrons (75-1200 nm), chylomicrons remnants (30-80 nm), very-low density lipoproteins (VLDL) (30-80 nm), intermediate-density lipoproteins (IDL) (25-35 nm), low-density lipoproteins (LDL) (18-35 nm), high-density lipoproteins (HDL) (5-12 nm) and lipoprotein (a) (Lp(a)) (~30 nm).

1.3.2 Exogenous lipoprotein pathway

After a meal, dietary triglycerides and cholesterol esters are hydrolyzed in the gastrointestinal tract by intestinal and pancreatic lipases. The produced monoglycerols, fatty acids and cholesterol are absorbed by the enterocytes. The enterocytes form new triglyceride and cholesterol esters which are packaged in chylomicrons in the intestinal ER. Every chylomicron particle contains also one APOB48 molecule, which is a truncated, post-translationally cleaved form of APOB100 produced by the intestine which does not bind LDL-receptors [43]. The chylomicrons are secreted into the lymph and, via the thoracic duct and systemic circulation, are delivered to the adipose tissue, the muscle and other tissues. Chylomicrons become the substrate of lipoprotein lipase (LPL), a highly expressed enzyme on the capillary endothelium [52]. This results in a significant decrease in the chylomicron size and formation of chylomicron remnants which, being poor in triglyceride but enriched in cholesterol esters, acquire APOE. Subsequently, the remnant particles are entirely taken up by the liver due to the binding of the APOE to the hepatocyte LDL-receptor via endocytosis, leading to their clearance from the circulation. Thus, the exogenous lipoprotein pathway provides an efficient delivery of dietary lipids to the periphery tissues while the produced cholesterol is transferred to the liver for utilization to VLDL or bile acids formation [49,50,53,54].

1.3.3 Endogenous lipoprotein pathway

A number of triglycerides and cholesterol esters, which are synthesized and stored in the liver, are subsequently used for the formation and secretion of the VLDL lipoproteins. The hepatocytes synthesize APOB100 and the Microsomal triglyceride transfer protein transfers the lipids from the cytosolic part of the ER to the lumen where the triglyceride-rich VLDL lipoprotein is generated [55].The VLDL is secreted via the Golgi apparatus [56] and transported to the peripheral tissues via the circulation where it gets hydrolyzed by the LPL in a similar way to the chylomicrons [52]. The removal of triglycerides from the VLDL particles gradually leads to the transformation of VLDL to APOE- and cholesterol ester-enriched IDL. A fraction of the IDL lipoproteins are removed from the circulation via binding of their APOE molecule to the liver LDL-receptor. The IDL that remains in the circulation is hydrolyzed by LPL, generating LDL. The LDL particle consists mostly of cholesterol ester and APOB100 and is taken up by the hepatocytes via LDL-receptor mediated endocytosis. The levels of LDL-receptors expressed on the hepatocyte surface are regulated by the cholesterol content of the cell. A decrease in the number or activity of the LDL-receptors leads to decreased LDL clearance, and thus to a higher LDL concentration in the circulation [49].

1.3.4 HDL metabolism and reverse cholesterol transport

HDL particles are characterized by high density and small size. Their surface coat is composed of apolipoproteins, non-esterified cholesterol and phospholipids while the core primarily contains cholesterol esters. However, the HDL compositions change and evolve as the HDL circulate in the plasma [49]. The main HDL structural protein (APOA1) is synthesized and secreted predominantly by the liver and the intestine and becomes lipidated with cholesterol and phospholipids; the primary lipids source is mostly identified from hepatocytes and enterocytes, as well as extrahepatic tissues such as myocytes and adipocytes. However, it has been observed that HDL can obtain cholesterol and phospholipids from chylomicrons and VLDL during their hydrolysis by the LPL. The cholesterol-rich HDL lipoprotein is mainly transferred to the liver where only the cholesterol compound is absorbed by the hepatocytes without internalization of the whole HDL particle. The cholesterol-poor HLD particle is subsequently released back into the circulation. Under some unknown conditions, Cholesterol ester transfer protein can catalyze cholesterol transfer from the HDL molecule to APOB containing particles resulting in triglyceride-rich HDL [57]. In conclusion, the reverse cholesterol transport plays a pivotal role in the cholesterol homeostasis and in protecting from the development of atherosclerosis [26,49,53,54,58–62] .

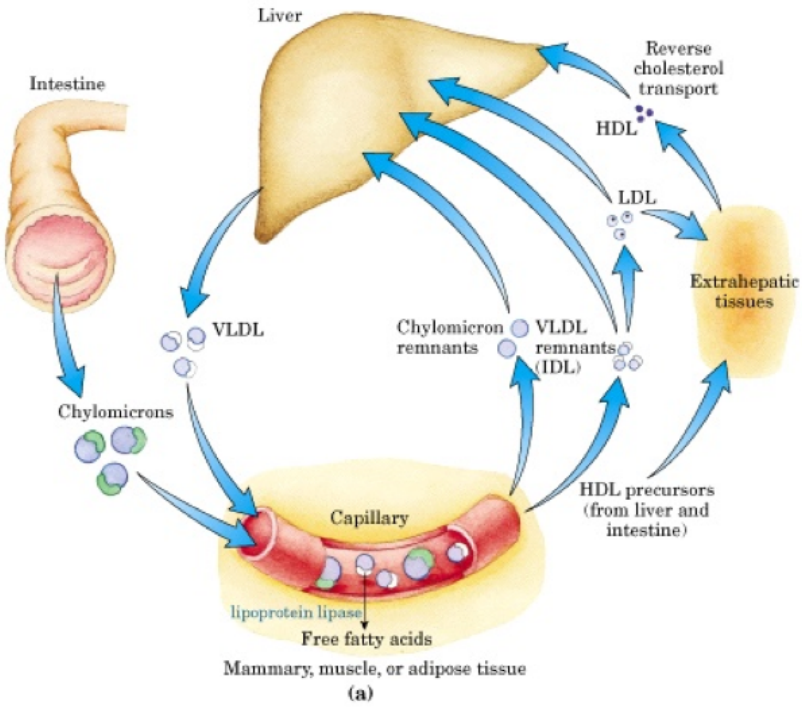

Figure 5. Endogenous and exogenous lipoprotein metabolism [135].

1.4 TRIGLYCERIDES AND CARDIOVASCULAR DISEASE

Cardiovascular disease (CVD) is the leading cause of morbidity, mortality and disability in industrialized countries and the prevalence of these diseases is increasing rapidly in developing countries, with approx. 17,5 millions of deaths worldwide in 2012 [63]. Coronary heart disease (CHD) is the most common type of CVD caused by atherosclerosis. Atherosclerosis is a chronic disease characterized by artery-wall thickening as a result of invasion and accumulation of cholesterol enriched-differentiated macrophages (foam cells) and proliferation of intimal-smooth-muscle cell. The created fibrofatty plaque disturbs the blood flow causing occlusive thrombosis with high possibility for tissue infarction [64]. Several excellent reviews have reported a range of atherogenic risk factors that give rise to increased CVD risks including male gender, age, ethnicity, socio-economic status, family history of CVD, smoking, alcohol consumption, type 1 and 2 diabetes mellitus, hypertension, obesity and disturbances of the plasma lipid profile [65].

Atherogenic dyslipidemia has been shown in population studies to be significantly associated with higher CVD risk [66 67] and consists of altered lipid profile in the blood circulation which involves the following conditions: increased LDL- and triglyceride concentration and decreased HDL concentration [68]. A major characteristic of atherogenic dyslipidemia is that it is found in patients with obesity, the metabolic syndrome, insulin resistance, and type 2 diabetes mellitus [69].

An important component of atherogenic dyslipidemia is the increased plasma triglyceride concentration. The secretion of large triglyceride-rich VLDL lipoproteins leads to reduced clearance of chylomicrons and chylomicron remnants due to competition for binding and triglyceride hydrolysis by lipoprotein lipase. The cholesterol-rich lipoprotein remnants are highly atherogenic, thus triggering the foam cell formation and substantially contributing to the cholesterol accumulation in the arterial wall, as reviewed in [70,71]. Additionally, the high triglyceride content of VLDL lipoproteins leads to triglyceride enrichment of LDL and HDL. This results in the accumulation of small, dense LDL and triglyceride-enriched HDL particles that are highly atherogenic. The LDL particles are susceptible to oxidation when present in the vessel wall, leading to the formation of the atherosclerotic plaque. Triglyceride-enriched HDL becomes substrate for lipolysis by hepatic lipase, leading to HDL catabolism and reduced reverse cholesterol transport. For this, there has been shown a positive association between the progression of CVD and the concentrations of triglyceride-rich lipoprotein remnants [72].

Triglyceride levels are highly dependent on genetic factors whereas several environmental factors, such as alcohol intake and diabetes, may increase hypertriglyceridemia. As reviewed in [73], the epidemiological associations between plasma triglyceride levels and CHD risk is not as strong as with LDL levels. However, genome-wide association (GWA) studies have lately provided genetic evidence that TRLs, as assessed by plasma triglycerides, represent a "casual" risk factor because of the linkage between single nucleotide polymorphisms (SNPs)

in genes modulating plasma triglyceride levels and CHD risk [73]. These studies have stimulated the interest in targeting triglyceride metabolism for therapeutic purpose.

Several GWA studies have identified a locus on chromosome 19p12, also-called NCAN locus, in association with plasma triglyceride and LDL levels. 19p12 locus was found to be associated with hepatic triglyceride content and non-alcoholic fatty liver disease [74,75]. Nevertheless, 19p12 locus consists of at least 19 different genes with the gene responsible for the reported associations not been identified at the beginning of this Thesis. The overall aim of the studies presented in this Thesis is to evaluate the functional roles of three proteins involved in hepatic triglyceride metabolism in the regulation of the TRL synthesis and secretion.

2 HYPOTHESIS AND AIMS

2.1 GENERAL HYPOTHESIS

The liver plays a major role in the regulation of TRL metabolism. A large number of proteins are involved in the control of different aspects of the hepatic triglyceride homeostasis, including triglyceride-synthesis, -storage, -hydrolysis and TRL secretion, but the specific roles of these proteins have not been analyzed in detail. The aim of this thesis is to functionally characterize three proteins (TM6SF2, PNPLA2 and ABHD5) that influence triglyceride metabolism in the human liver.

2.2 SPECIFIC AIMS

Paper I.

Hypothesis. GWA studies have identified a genetic locus (19p12) associated with both variations in the plasma triglyceride concentration and risk for NAFLD, but the gene/protein responsible for these relationships has not been uncovered. We hypothesized that this unknown protein plays a role in hepatic triglyceride metabolism, specifically in relation to TRL secretion and lipid-droplet metabolism.

Aim. To identify the gene in the 19p12 locus associated with plasma triglyceride concentrations and NAFLD and to investigate the phenotype of the protein in relationship with its hepatic expression, sub-cellular localization and impact on TRL secretion and lipid-droplet metabolism in human hepatoma cells.

Paper II.

Hypothesis. Three members of the PNPLA protein family, PNPLA2, PNPLA3 and PNPLA4 are expressed in human liver cells and are able to hydrolyze triglycerides as analyzed using *in vitro* systems. However, the physiological roles of these PNPLA proteins in hepatic triglyceride metabolism are largely unknown.

Aim. To investigate the physiological roles of PNPLA2, PNPLA3 and PNPLA4 proteins in hepatic triglyceride metabolism by studying the effects of gene-specific siRNA inhibition of these proteins on TRL-secretion and lipid-droplet metabolism in human hepatoma cells.

Paper III.

Hypothesis. ABHD5 is a co-activator of PNPLA2 in adipocytes, but the physiological role of ABHD5 in human hepatocytes has not been established. Moreover, there is evidence that

ABHD5 may exhibit a physiological function in hepatic triglyceride metabolism that is independent from PNPLA2.

Aim. To study the functional role of ABHD5 in hepatic triglyceride metabolism using gene-specific siRNA inhibition and to evaluate the relationship between ABHD5 and PNPLA2 with regard to TRL-secretion and lipid-droplet metabolism in human hepatoma Huh7 cells.

3 EXPERIMENTAL PROCEDURES

3.1 HUMAN COHORTS

For Paper I of this thesis, the human cohort derived from the Advanced Study of Aortic Pathology (ASAP) was used. This cohort is comprised of liver biopsies obtained from patients undergoing aortic valve surgery as described by [76]. All protocols were approved by the ethics committee of the Karolinska Institutet and informed consent was obtained from all participants according to the Helsinki Declaration. Gene expression of the liver samples was analyzed using the Affymetrix GeneChip Human exon 1.0 ST microarray.

3.2 CELL CULTURES

Human immortalized liver-derived cell lines HepG2 and Huh7 were used for the functional studies in all the papers, purchased from the American Type Culture Collection (HB- 8065) and the Health Science Research Resources Bank (cell no. JCRB0403; Osaka, Japan) respectively. Huh7 is a well-established and differentiated hepatocyte cell-line originally obtained from the liver of a 57-year-old male with a well-differentiated hepatocellular carcinoma in 1982 [77]. As a verification of our results, we repeated most of our experiments in another hepatocellular carcinoma cell line, HepG2, which has also been widely used in studies as a model of human triglyceride metabolism [78].

3.3 SMALL INTERFERING RNA (SIRNA)

Gene inhibition allows the functional study of a particular gene/protein using *in vitro* or *in vivo* models. This can be achieved thanks to RNA interference (RNAi), a mechanism which induces gene silencing by targeting complementary mRNA for degradation with double-stranded RNA (dsRNA) as reviewed in [79]. The experimental protocol involves transfection with either synthetic small interfering RNA (siRNA) probes or with plasmids coding for short-hairpin shRNA molecules that resemble intermediates of the microRNA pathway and become activated intracellularly [80]. In all our studies we used siRNA probes, obtained from a commercial supplier (Ambion), consisting of 20–30 nucleotides to reduce the expression of the selected target-genes.

siRNA transfection requires “vehicles” which will deliver the siRNA to the appropriate site of action inside the cell. The delivery of such molecules is either accomplished by transfection using cationic liposomes, electroporation or by viral mediated delivery. In order to avoid the cytotoxicity caused by electroporation or the changes in the cell-host genome caused by viral-mediated techniques, the delivery method of our experimental approach was lipofection. In this procedure, cationic lipid-based particles interact electrostatically with the

siRNA and they form coated vesicles, the “lipoplexes”, with the nucleic acid in their core. The lipoplexes bind to the cell membrane and release their content in the cytoplasm. When siRNAs enter the cell, they bind to a multiprotein component complex known as RISC (RNA induced silencing complex) which becomes aligned on the target mRNA, thus leading to mRNA cleavage [81,82].

3.4 RNA EXTRACTION AND SYNTHESIS OF CDNA

RNA was extracted from the cells using E.Z.N.A. Total RNA Kit 1 (Omega Bio-tek), followed by quantification of the RNA concentration by Nanodrop1000 spectrophotometer (Thermo Fisher Scientific). cDNA was synthesized with a High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific) and PCR amplification.

3.5 GENE EXPRESSION ANALYSIS

We used Taqman primer probes and AB7500 sequence detection system (Applied Biosystems) to analyze the relative mRNA expression of selected genes. The Ct (threshold cycle number) values were obtained to assess the relative gene expression using the comparative ΔΔCT method and subsequently adjusting for the endogenous control RPLP0. The results were verified using the relative standard curve method according to the Applied Biosystems guidelines.

3.6 IMMUNOBLOT ANALYSIS

Immunoblotting was performed in order to validate the efficacy of siRNA transfection and to evaluate the level of protein reduction of the gene-targets in our studies. Proteins were extracted using RIPA buffer and immunoblots were performed as described in detail in every manuscript. The antibodies that were used are listed in the respective manuscript and were validated in previous studies. The visualization of the blots was performed by using a LAS-1000 Imager (Fujifilm) while the intensities of the protein bands were measured by ImageJ software. Beta-actin was used as an endogenous control for normalization.

3.7 CELL STAINING

The subcellular localization of a protein of interest can be analyzed using fluorescent proteins available for microscopy with fluorescent- and confocal-microscopes. The protein of interest is cloned into a vector (plasmid) encoding a fluorescent tag. The cells are transfected with the vector and the fluorescent protein fusion is expressed within the target cell, thus making it possible to track the protein of interest. In Paper I we purchased from OriGene full-length

human probes for TM6SF2 and CALR proteins which were GFP-tagged or FP635- tagged in their C-terminal. The details of the plasmids, cellular transfection and the lipid-delivery can be found in the Material and Methods section of Paper I.

The protein visualization with the plasmid transfection method has a number of disadvantages, including the possibility that the fused protein oligomerizes, becomes charged or changes its actual molecular size. It is thus possible that overexpression of a large or charged protein-construct can change the subcellular localization of the protein of interest [83]. In addition, it is conceivable that protein overexpression generates an 'overload' of intra-cellular protein, leading to the accumulation of the tagged protein at non-physiological sites.

In order to deal with these problems, we visualized the proteins of interest in Papers II and III using fluorescently-labelled monoclonal antibodies. The use of monoclonal antibodies offers the advantage of high specificity against their protein-target as compared to polyclonal antibodies. However, minor changes in the structure of the antigen epitope due to the cell fixation or processing can affect the function of the monoclonal antibodies [84]. Thus, for every study the appropriate monoclonal antibody as well as the cell-fixation method was selected with care.

For visualization of the lipid droplets we used either Bodipy 493/503 or HCS LipidTox Red purchased from Life Technologies and Invitrogen, respectively. Both fluorochromes stain the lipid droplets in the cells thanks to their lipophilic nature and their specificity with neutral lipids [85,86]. DAPI (4′, 6-diamidino-2-phenylindole), supplied by Vector Laboratories, was used for the staining of the cell nucleus. The protocols for cell fixation, permeabilization and staining with fluorescent dyes or antibodies are described in detail in Paper II and Paper III.

3.8 CONFOCAL MICROSCOPY

For visualization of the fluorescence-labelling we used a Leica SP5 inverted confocal microscope equipped with a 63×1.4 NA oil lens (Leica Microsystems). We obtained image z-stacks consisting of multiple optical slices taken at 0.2-0.5 μm intervals. The details of the confocal microscopy as well as the analysis of the images can be found in the Materials and Methods section of every Paper.

3.9 C14-LABELLING

Fatty acids and glycerol are the "building blocks" for the hepatic triglyceride synthesis and predominantly derive as from triglyceride hydrolysis by adipose tissue [87]. The process of the triglyceride metabolism in the hepatoma cell, from fatty acid esterification to the triglyceride secretion, can be traced by using triglyceride precursors labelled with radioisotopes. The use of stable isotope-labeled tracers allows for the quantitative evaluation of major pathways of the fatty acid and triglyceride metabolism *in vivo*.

In all our Papers, we analyzed triglyceride secretion by incubation of the hepatoma cells with C^{14}-labelled glycerol. This semi-quantifying method allowed us to trace the C^{14}-labelled triglyceride that was synthesized and to measure the secreted triglyceride in the cell-culture medium. In another series of experiments, we treated our hepatoma cells with C^{14}-labelled palmitic acid as a labelled source of fatty acids. Palmitic acid was employed for the evaluation of the effect of siRNA silencing on fatty acid uptake by the cells, as well as on the synthesis and hydrolysis of triglycerides as analyzed in detail in Papers II and III.

3.10 LIPID EXTRACTION

For the extraction, isolation and quantification of the radio-labelled triglycerides, we used a two-phase separation method. A small amount of the cell-lysate or the cell medium was added to a mixture of Isopropanol-Hexane (4:1) and incubated for 30 minutes, followed by addition of 500 µl of Hexane-Diethyl ether (1:1). After 20 minutes incubation, the bottom phase is removed and dried. The sample was dissolved in Heptane and analyzed by thin layer chromatography (TLC). The C^{14}-labelled triglycerides were quantified using a scintillation counter.

3.11 STATISTICS

In all the Papers, the analysis and visualization of all the *in vitro* data was executed by using the GraphPad Prism software. Differences in continuous variables between groups were tested by Student´s t test.

In Paper I the association between mRNA expression and the SNPs was tested by multiple linear regression and the correlation between mRNA expression and the SNP genotypes was examined by ANOVA analysis.

Level of significance was set to $p<0.05$ and Bonferroni correction was used to adjust the threshold p-values for multiple testing where appropriate.

4 RESULTS

4.1 PAPER I. TM6SF2 IS A REGULATOR OF LIVER FAT METABOLISM INFLUENCING TRIGLYCERIDE SECRETION AND HEPATIC LIPID DROPLET CONTENT

Identification of TM6SF2 as the putative causal gene responsible for the observed relationship with triglyceride concentration in genome-wide association studies

Genome-wide association studies have shown an association between the 19p12 locus and plasma triglyceride concentration, but the identity of the hepatic gene responsible for this association was unknown [88–90]. We analyzed the hepatic expression of the 19p12 locus genes and the lead SNP rs10401969 in human liver biopsies and observed a positive correlation between hepatic *TM6SF2* mRNA levels and plasma triglyceride concentration. This suggests that *TM6SF2* is the putative gene in the 19p12 locus involved in triglyceride metabolism.

Subcellular localization of TM6SF2 in human Huh7 hepatoma cells.

Protein pattern and domain prediction software predicted 7-10 transmembrane domains for TM6SF2, indicating that TM6SF2 is a membrane protein. This made us hypothesize that TM6SF2 is located in ER. We used GFP-tagged overexpression vector for TM6SF2 in combination with FP635-tagged overexpression vector for CALR (an ER marker) to evaluate the co-localization of TM6SF2 with the ER *(Figure 6)*.

Figure 6. TM6SF2 is localized in the endoplasmic reticulum.

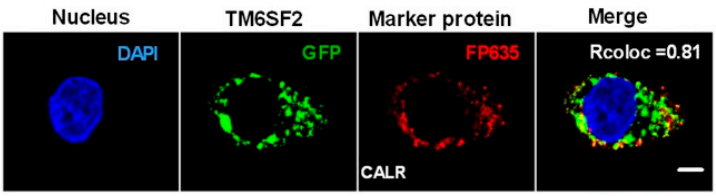

Figure 6. Co-localization analysis of TM6SF2 (green) with CALR (red) in Huh7 hepatoma cells. Co-localization was quantified using Pearson correlation (Rcoloc) and represents mean values of one to three cells evaluated in 4-6 independent experiments. Scale bar, 10 μm.

We observed a high co-localization of TM6SF2 with CALR with Rcoloc values of 0.83 ± 0.04 (mean ± SD). As shown in Figure 2B of Paper I, comparable co-localizations of TM6SF2 with Alexa 633-tagged monoclonal antibodies targeting the ER-marker Protein Disulfide Isomerase (PDI), with Rcoloc values of 0.78 ± 0.05, or the ER-Golgi intermediate compartment protein 2 (ERGIC), with Rcoloc values of 0.80 ± 0.03, were found. In contrast, very low co-localization was observed between TM6SF2 and the Golgi complex marker Giantin, with Rcoloc values of 0.30 ± 0.08. Overall, this analysis indicates that TM6SF2 is primarily localized in the ER.

TM6SF2 influences the secretion of triglyceride-rich lipoproteins and the triglyceride content of cellular lipid-droplets.

We subsequently performed *TM6SF2* siRNA silencing experiments and observed that *TM6SF2* silencing led to decreases in *TM6SF2* mRNA and TM6SF2 protein levels in both Huh7 and HepG2 hepatoma cells (Figure 3A-D, Paper I). We also observed a decrease in the secretion of TRLs following *TM6SF2* siRNA inhibition (*Figure 7*).

Figure 7. *TM6SF2* siRNA inhibition reduces the secretion of triglycerides and APOB by human hepatoma cells.

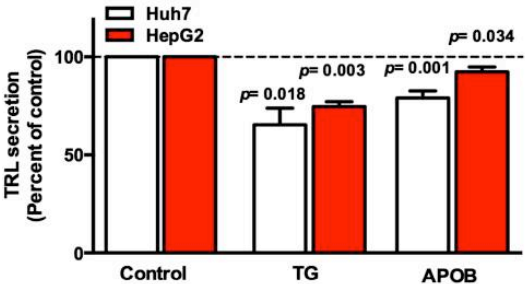

Figure 7. Effect of *TM6SF2* siRNA inhibition on secretion of triglycerides (TG) and apolipoprotein B (APOB) by Huh7 and HepG2 hepatoma cells. The values are expressed as percent of control experiments, indicated with a dotted line. Values represent mean ± SD of 6-8 independent experiments. Differences were determined using Student's t test.

Additionally, *TM6SF2* siRNA silencing led to an increase of lipid-droplet content of human hepatoma Huh7 cells, as measured both by an *in vitro* method (*Figure 8A*) and by confocal microscopy (*Figures 8B and 8C*). Similar effects were observed following *TM6SF2* silencing of HepG2 cells (Figure 4E-G, Paper I). Moreover, overexpression of FP635 tagged-TM6SF2 resulted in reduced triglyceride content of Huh7 hepatoma cells as compared to cells not overexpressing TM6SF2 (Figure 5, Paper I). Overall, these *TM6SF2* siRNA silencing and overexpression experiments point to opposing effects of the TM6SF2 concentration on the secretion of TRLs and the accumulation of triglycerides in lipid-droplets of the human hepatoma cells.

Figure 8. *TM6SF2* siRNA silencing reduces cellular triglyceride content and lipid-droplet area of human hepatoma cells.

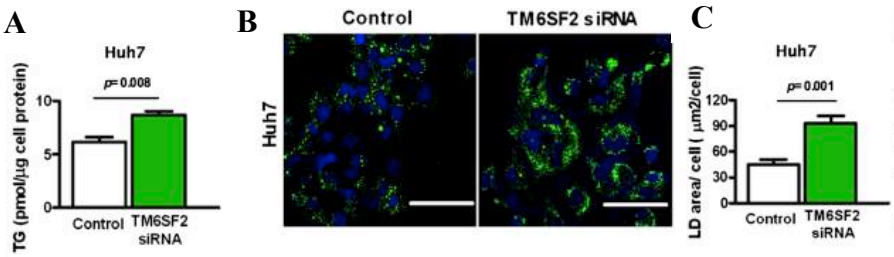

Figure 8. Effect of *TM6SF2* siRNA inhibition on cellular triglyceride (TG) content (A) and lipid-droplet area analyzed by confocal microscopy (B and C) of Huh7 cells. The results are expressed as mean ± SD, n = 6-8, Scale bar 75 µm. Differences were determined using Student's t test.

4.2 PAPER II. PNPLA2 INFLUENCES SECRETION OF TRIGLYCERIDE-RICH LIPOPROTEINS BY HUMAN HEPATOMA CELLS

PNPLA2, but not PNPLA3 or PNPLA4, influences cellular triglyceride hydrolysis and TLR secretion, but not lipid-droplet homeostasis of human hepatoma cells.

We performed gene-specific siRNA silencing experiments in human hepatoma Huh7 and HepG2 cells to evaluate the physiological function of PNPLA2, PNPLA3 and PNPLA4 in hepatic triglyceride metabolism. Gene-specific siRNA inhibition led to significant decreases of mRNA and protein levels of the respective genes (Figure 1A-C, Paper II). We subsequently measured triglyceride hydrolysis activity and observed that *PNPLA2* siRNA silencing significantly reduced triglyceride hydrolysis while *PNPLA3* and *PNPLA4* siRNA silencing had no effect on triglyceride hydrolysis (*Figure 9A*).

Figure 9. *PNPLA2* siRNA inhibition reduces triglyceride hydrolysis and decreases TRL secretion but does not affect cellular triglyceride accumulation.

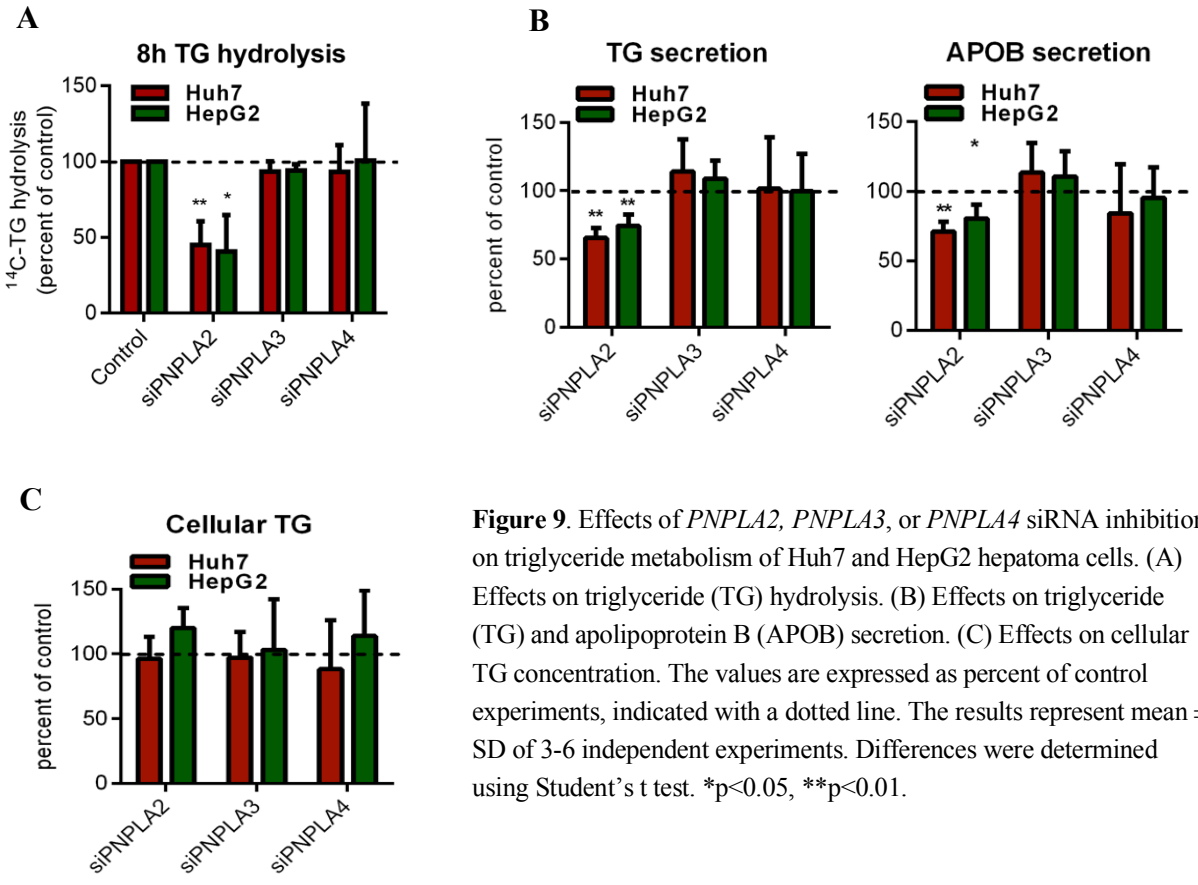

Figure 9. Effects of *PNPLA2, PNPLA3*, or *PNPLA4* siRNA inhibitions on triglyceride metabolism of Huh7 and HepG2 hepatoma cells. (A) Effects on triglyceride (TG) hydrolysis. (B) Effects on triglyceride (TG) and apolipoprotein B (APOB) secretion. (C) Effects on cellular TG concentration. The values are expressed as percent of control experiments, indicated with a dotted line. The results represent mean ± SD of 3-6 independent experiments. Differences were determined using Student's t test. *p<0.05, **p<0.01.

Moreover, siRNA silencing of *PNPLA2* was associated with reduced triglycerides and APOB secretions (*Figure 9B*). In contrast, no effects of *PNPLA3* or *PNPLA4* siRNA inhibitions on TRL secretion were observed (*Figure 9B*). Thus, *PNPLA2* siRNA inhibition reduced triglyceride hydrolysis and TRL secretion in Huh7 and HepG2 cells, while no effects of *PNPLA3* or *PNPLA4* siRNA inhibitions on these functions were observed.

We subsequently analyzed the effects of *PNPLA2*, *PNPLA3* and *PNPLA4* siRNA inhibition on cellular triglyceride content. To our surprise, we found that the cellular triglyceride concentrations of the Huh7 and HepG2 cells were not affected by either *PNPLA2*, *PNPLA3*, or *PNPLA4* siRNA inhibitions (*Figure 9C*). Moreover, no effects of *PNPLA2* siRNA inhibition on lipid-droplet area/cell or lipid-droplet size-distribution was observed in Huh7 and HepG2 cells analyzed by confocal microscopy (Figure 3, Paper II). Overall, the gene-specific siRNA inhibition experiments suggest that PNPLA3 and PNPLA4 do not influence hepatic triglyceride metabolism. However, *PNPLA2* siRNA inhibition was associated with reduced TRL secretion, while no effects on lipid-droplet metabolism were observed.

Atglistatin inhibition of PNPLA2 and PNPLA2 siRNA inhibition have similar effects on triglyceride metabolism of human hepatoma cells.

We used Atglistatin, a pharmacological inhibitor of PNPLA2, to verify the results from the *PNPLA2* siRNA experiments described above. It was found that Atglistatin treatment, like *PNPLA2* siRNA inhibition, was associated with reduced triglyceride hydrolysis activity in both Huh7 and HepG2 cells (Figure 5A, Paper II). No evidence was found that a combination of the two PNPLA2 inhibition methods leads to a greater inhibition of total cellular triglyceride-hydrolase activity as compared to *PNPLA2* siRNA inhibition alone (Figure 5A, Paper II).

Figure 10. Comparable effects of *PNPLA2* siRNA silencing, Atglistatin treatment or the combination of *PNPLA2* siRNA silencing and Atglistatin on triglyceride metabolism of Huh7 and HepG2 hepatoma cells.

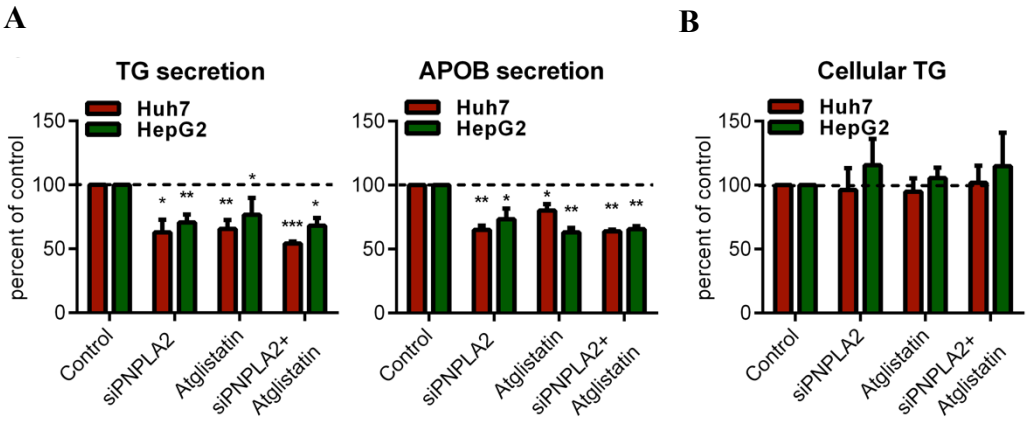

Figure 10. Atglistatin effects on triglyceride metabolism. (A) Effects on triglyceride (TG) and apolipoprotein B (APOB) secretion. (B) Effects on cellular TG concentration. The values are expressed as percent of control experiments, indicated with a dotted line. The results represent mean ± SD of 3-6 independent experiments. Differences were determined using Student's t test. *p<0.05, **p<0.01, ***p<0.001.

Atglistatin inhibition of PNPLA2 and the combination of Atglistatin and *PNPLA2* siRNA inhibitions were associated with reduced triglyceride and APOB secretions (*Figure 10A)*. No evidence was found that a combination of the two PNPLA2 inhibition methods leads to a greater reduction of TRL secretion as compared to *PNPLA2* siRNA inhibition alone. Additionally, no effects were observed of Atglistatin inhibition or the combination of Atglistatin and *PNPLA2* siRNA inhibitions on the cellular triglyceride concentrations (*Figure 10B*). In short, comparable effects of Atglistatin inhibition and *PNPLA2* siRNA inhibition on triglyceride metabolism were observed in both hepatoma cell-lines. Moreover, no evidence was found for additive effects of the two PNPLA2 inhibition methods on hepatic triglyceride metabolism.

PNPLA2 is not associated with lipid-droplets

The absence of an effect of PNPLA2 inhibition on lipid-droplet metabolism raised the question as to the subcellular localization of the PNPLA2 protein. We therefore investigated the association of PNPLA2 with lipid-droplets using confocal microscopy, after staining of the Huh7 cells with a human monoclonal PNPLA2 antibody and LipidTox Red (*Figure 11*). It was found that PNPLA2 protein is primarily present in the cellular cytoplasm, while no enrichment of PNPLA2 was found around the lipid droplets, independently of whether the cells were cultured in 10% FBS cell medium or oleate-supplemented cell-medium. Similar observations were made when the subcellular localization of PNPLA2 was analyzed in HepG2 cells (Figure 6, Paper II).

Figure 11. PNPLA2 is not associated with lipid-droplets in Huh7 hepatoma cells.

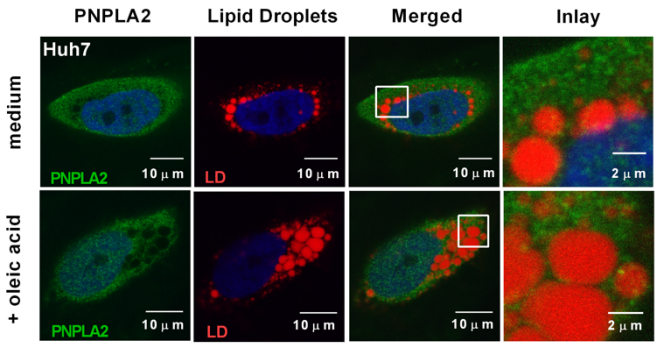

Figure 11. Representative confocal microscopy images of co-localization analysis of PNPLA2 (green) with lipid droplets (red) in Huh7 cells incubated with FBS-supplemented medium (upper lane) or in 0.4 mM oleate-supplemented medium (lower lane).

PNPLA2 shows co-localization with the endoplasmic reticulum

The observed "cytoplasmic" localization of PNPLA2 (*Figure 11*) is surprising and may be due to its proximity to the cellular compartment responsible for TRL synthesis. We visualized the lipid-droplet surface and the ER by employing Perillipin2 (PLIN2) and Protein disulfide isomerase (PDI) human monoclonal antibodies, respectively (Figures 7A and 7B, Paper II). It was found that PNPLA2 is partially co-localized with PLIN2, with Rcoloc values (mean ± SD) of 0.66 ± 0.02 in HepG2 cells and of 0.49 ± 0.07 in Huh7 cells (Figure 7A, Paper II*)*. The cytoplasmic distribution pattern of PDI and PNPLA2 showed considerable overlap, with Rcoloc values of 0.81 ± 0.05 and 0.61 ± 0.06 for HepG2 and Huh7 cells, respectively (*Figure 12*).

Figure 12. Co-localization of PNPLA2 with the endoplasmic reticulum.

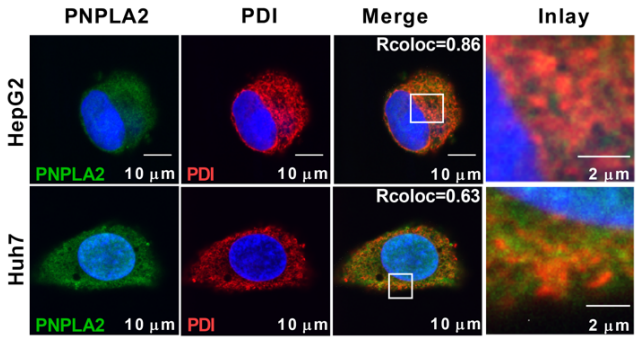

Figure 12. Representative confocal microscopy images of PNPLA2 (green) visualizing co-localization with PDI (red) in HepG2 and Huh7 cells. Cells were stained with Alexa Fluor 488- and Alexa Fluor 594-labelled monoclonal antibodies. Colocalization was quantified using Pearson correlation (Rcoloc)

4.3 PAPER III. THE PNPLA2 CO-ACTIVATOR ABHD5/CGI-58 INFLUENCES THE SECRETION OF TRIGLYCERIDE-RICH LIPOPROTEINS IN HUMAN HEPATOMA CELLS

ABHD5 siRNA inhibition reduces triglyceride hydrolysis and TLR secretion without affecting cellular triglyceride content of Huh7 cells.

We evaluated the functional role of ABHD5 in hepatic triglyceride metabolism using gene-specific inhibition of *ABHD5* and *PNPLA2* in the human hepatoma Huh7 cell-line. *ABHD5* siRNA inhibition led to significant reductions of *ABHD5* RNA and ABHD5 protein concentrations (Figure 1A and 1B, Paper III). *ABHD5* siRNA inhibition reduced triglyceride hydrolysis and this effect was comparable to the effect of *PNPLA2* siRNA inhibition (*Figure 13*). The combined *ABHD5* and *PNPLA2* siRNA inhibition showed no additive effect on triglyceride hydrolysis. Moreover, *ABHD5* siRNA inhibition was associated with reductions of triglyceride and APOB secretion. Comparable decreases in TRL section were observed following *PNPLA2* siRNA inhibition and the combined *ABHD5* and *PNPLA2* siRNA inhibition. In contrast, the cellular triglyceride concentrations were not influenced by *ABHD5* siRNA inhibition, *PNPLA2* siRNA inhibition or the combined *ABHD5* and *PNPLA2* siRNA inhibition. The results of these inhibition experiments indicate that ABHD5 and PNPLA2 exhibit comparable effects on triglyceride metabolism in human hepatoma Huh7 cells.

Figure 13. Comparable effects of *ABHD5* and *PNPLA2* siRNA inhibition on triglyceride metabolism of Huh7 cells.

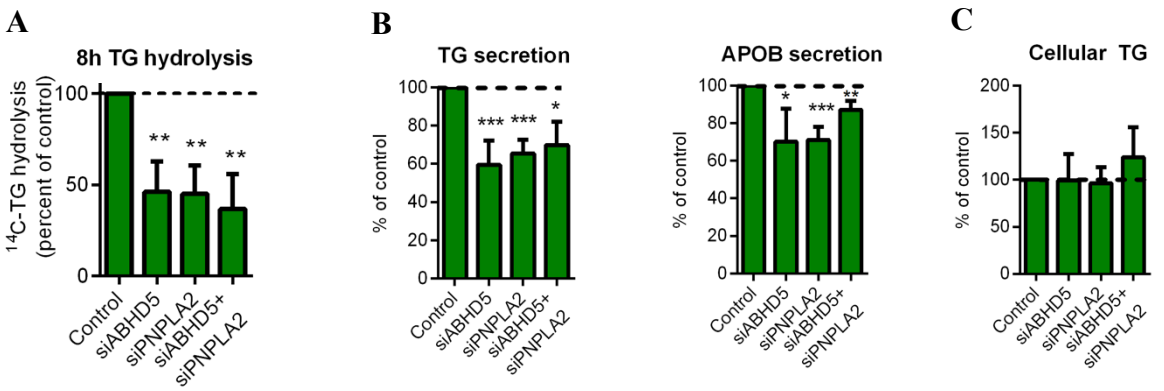

Figure 13. Effects of *ABHD5* and *PNPLA2* siRNA inhibition in hepatic triglyceride metabolism. (A) Effects on cellular triglyceride (TG) hydrolysis. (B) Effects on TG and apolipoprotein B (APOB) secretion. (C) Effects on cellular TG concentration. The values are expressed as percent of control experiments, indicated with a dotted line. The results represent mean ± SD of 3-6 independent experiments. Differences were determined using Student's t test. *p<0.05, **p<0.01, ***p<0.001.

ABHD5 is not associated with lipid droplets.

In Study II we reported that PNPLA2 is not associated with cellular lipid-droplets of human hepatoma cells. The question was raised as to whether ABHD5 is also absent from lipid-droplets in Huh7 hepatoma cells. We employed confocal microscopy to evaluate this question, following the staining of Huh7 cells with a human monoclonal ABHD5 antibody and LipidTox Red. As shown in *Figure 14*, ABHD5 protein is primarily found in the cytoplasm and not around the lipid droplets, a distribution pattern that is similar to the subcellular localization of PNPLA2 in hepatoma cells (Figure 3, Paper III).

Figure 14. No evidence for co-localization of ABHD5 with lipid droplets.

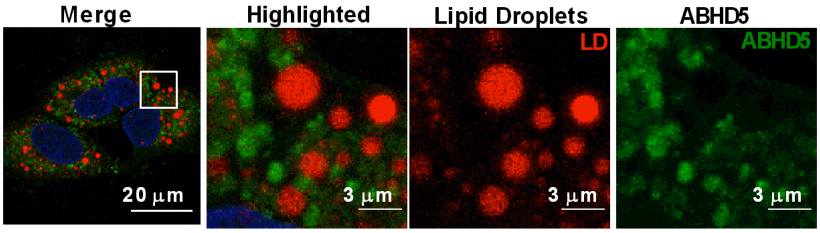

Figure 14. Representative confocal microscopy images of co-localization analysis of PNPLA2 (green) with lipid droplets (red) in Huh7 cells incubated with FBS-supplemented medium.

ABHD5 is co-localized with PNPLA2 and associated with the endoplasmic reticulum

The functional analysis and the subcellular localization studies suggested that ABHD5 and PNPLA2 collaborate in the regulation of TRL secretion by human hepatocytes. We performed co-localization studies to substantiate that the two proteins are present in human hepatoma cells in close proximity to one another. ABHD5 and PNPLA2 were stained with specific human monoclonal antibodies and the degree of co-localization of the two proteins was analyzed using confocal microscopy. As shown in *Figure 15*, a high degree of co-localization of ABHD5 with PNPLA2 was observed in human Huh7 hepatoma cells, with average Rcoloc values (mean ± SD) of 0.84 ± 0.08. Overall, the co-localization studies indicate the close proximity of ABHD5 with PNPLA2.

Figure 15. Co-localization of ABHD5 and PNPLA2.

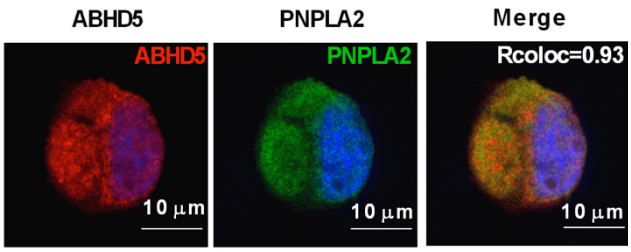

Figure 15. Representative confocal microscopy image of Huh7 hepatoma cells stained with Alexa Fluor 594 - labelled ABHD5 (red) and Alexa Fluor 488-labelled PNPLA2 (green) antibodies. Colocalization was quantified using Pearson correlation (Rcoloc) and represents mean values of one to three cells evaluated in 3-4 independent experiments.

5 DISCUSSION

In this section of the thesis I will discuss a number of issues and results which were not addressed in the Discussion sections of Papers I, II, and III.

5.1 SELECTION OF A MODEL SYSTEM FOR THE STUDY OF HEPATIC TRIGLYCERIDE METABOLISM

The ideal, "golden standard" model for studying triglyceride metabolism in the human liver is by using primary human hepatocytes. Freshly isolated human hepatocytes secrete nascent TRLs within the VLDL size-range, similar to the secreted lipoproteins found in human serum [91]. However, fresh human liver samples are rarely available and the subsequent isolation of hepatocytes is dependent on delicate isolation procedures. Their limited life span, combined with phenotypic instability and differentiation within hours after their isolation makes primary hepatocytes a challenging study model. Additionally, it is notoriously difficult to perform siRNA transfection on primary human hepatocytes because they do not easily absorb the lipid complexes required for the transfection, as reviewed in [92].

Recent advances in maintaining the human liver morphology and specific functions of isolated primary human hepatocytes during standard cell culture include the co-culture with other cell-types, such as with endothelial cells or 3T3-J2 fibroblasts [93]. Additionally, formation of 3D-like structures, such as 3D-spheroid cultures, has demonstrated the potential to closely mimic human liver function *in vitro* discovery [94]. However, this type of pilot modelling systems are characterized by variability between cell-batches originating from different donors and lack of established experimental standards [95,96]. Addition of chemicals in the cell-culture media has been shown to achieve long-term functional maintenance of primary hepatocytes, thus opening new horizons for an efficient *in vitro* model for investigation of human hepatic metabolism [97].

Animal models have been used extensively to study hepatic triglyceride metabolism[98]. Unfortunately, often-used rodent models like mouse and rat show only limited translatability as regards human hepatic triglyceride metabolism. An example of the marked difference between rodents and humans in hepatic physiology is the secretion of two different isoforms of APOB, termed APOB100 and APOB48, by the murine liver, while human hepatocytes only secrete TRLs containing APOB-100 [44,99]. It is likely that different processes regulate the synthesis of the two types of TRLs secreted by rodent hepatocytes, but this question has, to the best of my knowledge, not been analyzed in any detail. In addition, it is conceivable that there are fundamental differences in the synthesis and secretion of APOB-100 containing TRLs by human and rodent hepatocytes, but this possibility is rarely considered.

Cell-culture systems are often used for functional and mechanistic studies because they offer possibilities that are difficult or impossible to achieve using *in vivo* models. Cell-lines offer the advantage of a cheap and flexible study system whose parameters, such as growth and culture media, can be easily adjusted. In this Thesis we used Huh7 and HepG2 hepatoma cell-lines. Unfortunately, there are some differences between these cell-lines and *in vivo* models; Huh7 and HepG2 cells secrete dense, relatively poorly-lipidated lipoproteins in the size - range of IDL-LDL, in contrast to the VLDL particles secreted *in vivo* by the human liver [100,101]. It is generally assumed that this phenomenon is a question of a reduced "thermostat" for TRL-secretion in human hepatoma cells, not a matter of a totally different mechanism of TRL-secretion. Several other human hepatoma cell-lines are also available, but none of these cell-lines have been used and analyzed to the same extent as the Huh7 and HepG2 cell-lines.

The low rate of secretion of TRLs by Huh7 and HepG2 cells creates challenges for the quantification of TRL secretion. We therefore used in all of our studies two different parameters to measure TRL secretion, quantifying the secretion of both triglycerides and APOB.

While differences of some metabolic features between Huh7 and HepG2 cells have been documented [92,102], we have tried as much as possible to perform duplicate experiments in both cell-lines in Papers I and II whereas in Paper III we used only Huh7 cells. Overall comparable results were obtained in Papers I and II as regards the analysis of hepatic triglyceride metabolism in the two cell-lines. Nevertheless, some minor, quantitative differences between the two cell-lines were observed. For example, in Paper I the effect of *TM6SF2* siRNA silencing on intracellular triglyceride concentrations was slightly stronger in HepG2 cells as compared to Huh7 cells, as investigated with both the *in vitro* triglyceride assay and confocal microscopy. In addition, the effects of *TM6SF2* siRNA inhibition (Paper I) and *PNPLA2* siRNA inhibition (Paper II) on TRL secretion was greater in Huh7 cells as compared to HepG2 cells. It is in this respect noteworthy that the difference in the effects of *PNPLA2* siRNA inhibition on TRL secretion between the two cell-lines was not influenced by the addition of oleic acid to the cell-culture medium (Paper II). Finally, in agreement with previous reports, we observed that Huh7 cells exhibited generally higher triglyceride and APOB secretion rates as compared to HepG2 cells [102,103]. In short, the use of the Huh7 and HepG2 cell-lines allowed us to perform siRNA inhibition experiments with high efficacy. Overall, we observed consistent qualitative effects of the gene-specific inhibitions of TM6SF2 and PNPLA2 on hepatic triglyceride metabolism.

5.2 THE USE OF OLEIC ACID

Under fasting conditions, circulating fatty acids are absorbed by the liver and stored in the lipid-droplets as triglycerides. Accumulation of excessive amounts of triglycerides results in hepatic steatosis, ultimately leading to NAFLD [104,105]. The main metabolic aspects associated with hepatic steatosis are cellular dysfunction and apoptosis [106,107]. Oleic acid is the most abundant non-saturated fatty acid present in circulation and has been used in numerous studies as an *in vitro* model of hepatic steatosis. Addition of exogenous unsaturated fatty acids, in the form of oleic acid, in the cell-culture media leads to an increase of the number and size of lipid-droplets in human Huh7 and HepG2 cell-lines, without induction of apoptosis [78,91,108]. Incubation with oleic acid also enhances the triglyceride and APOB secretion by both cell-lines [100,101,103] and promotes a slight density shift of secreted lipoproteins from the LDL size-range to the VLDL size-range [103,109]. In contrast, incubation of hepatoma cells with saturated fatty acids, such as palmitic acid, has been shown to induce cellular toxicity, apoptosis and cell death, accompanied by poor incorporation of the saturated fatty acids into lipid droplets [110,111].

Previous studies have employed oleic acid concentrations between 0.4 mM and 0.8 mM, with incubation times ranging from 3 to 24 hours [112–115]. The question was raised as to whether the relatively high oleate concentrations are required for these studies since they may possibly induce cellular toxicity. We therefore compared in Paper II the effect of 0.2 mM oleate-BSA conjugate-containing medium to FBS-containing medium in both Huh7 and HepG2 cells. Despite the relatively low oleate concentration, we confirmed the oleate effect on cellular triglyceride accumulation and TRL secretion. Meanwhile, no cell death was observed as measured by total cellular protein (unpublished data). Concentration of 0.2 mM oleic acid was sufficient to generate an increase of triglyceride and APOB secretion by more than 100% in both cell lines (Figure 4A, Paper II), comparable to the effect of 0.6 mM oleate employed in previous studies [103,109]. In conclusion, the oleate concentration of 0.2 mM was sufficient to stimulate the steps of lipoprotein metabolism that we evaluated in Paper II.

5.3 THE USE OF SIRNA

One of the experimental approaches used in this Thesis to investigate the function of proteins was the loss-of-gene function analysis via RNA interference (RNAi). As described in the section Experimental Procedures, RNAi employs either double-stranded siRNA or vector-based shRNA molecules. Previous studies in our lab have shown a lower silencing efficiency of shRNA transfection, combined with cytotoxic effects, as compared to siRNA transfection (unpublished data). It is for these reasons that we favor the use of the siRNA technique. However, the effect of siRNA is transient; the reduction of the target-protein is brief and starts to diminish 48 and 72 hours after transfection [116] . It is for this reason that we performed the metabolic analysis within 48 hours after the gene-specific silencing. In addition, siRNA inhibition is a knock-down method, not a knock-out procedure. Thus, there

always remains some non-degraded mRNA, especially in hard-to-transfect cell-lines, which may be enough for the maintenance of basic cellular functions.

We found no evidence of cellular cytotoxicity or cell death caused by transfection-reagent lipofectamine in our experiments, as analyzed by microscopy or measured by the amount of total cell protein. In Paper II we consistently observed 75-90% reductions of mRNA levels after gene-specific siRNA inhibitions of *PNPLA2, PNPLA3* and *PNPLA4*. In Paper III we obtained nearly 90% reductions of *ABHD5* mRNA concentration following *ABHD5* siRNA inhibition. It was reported that HepG2 cells are more difficult to transfect as compared to Huh7 cells because they exhibit a high proliferation rate; in every cell-division cycle the "lipoplex" (siRNA-containing vesicle) dissociates from the cell-membrane, thus resulting in a reduction of the inhibition efficiency [82]. Nevertheless, in our experiments we observed no consistent difference in the siRNA knock-down efficiency between Huh7 and HepG2 cells.

Finally, the introduction of siRNA probes can lead to the unintended knockdown of genes not being directly targeted. This phenomenon is called "off-target" effect and can lead to unexpected results as reviewed in [117]. In order to avoid "off-target" effects as much as possible, we tested in Papers II and III two different siRNA assays for every different knocked-down gene and compared their results (unpublished data). Overall, these consistent results indicate that there were limited "off-target" effects after employing transient siRNA techniques whereas the inhibition efficiency of the gene-specific silencing was more than 80%.

5.4 INTERPRETATION OF SUBCELLULAR LOCALIZATION STUDIES

Subcellular localization analysis provides information regarding the potential mechanistic role of a protein in cellular metabolism. Protein visualization and co-localization analysis with marker proteins of cellular compartments helps to uncover possible relations and interactions between proteins. From an experimental point of view, this approach involves the visualization of the target protein and/or organelle with either the (over-)expression of a fluorescence-tagged protein-construct or the staining with an antibody. In Paper I, we employed both methods to evaluate the role of TM6SF2 in hepatic triglyceride metabolism.

During the course of the TM6SF2 studies, we became aware of two limitations regarding the use of the plasmid overexpression method for metabolic analysis in human hepatoma cells. First, we consistently found that only a small number (usually <5%) of all hepatoma cells expressed the protein-construct encoded by the plasmid, presumably because of the low transfection efficiency of plasmids in the hepatoma cells. Secondly, we noted remarkable variation in the overall expression-degree of the protein-constructs in the transfected cells. Some cells expressed low levels of protein construct and exhibited a normal cell-morphology when examined by confocal microscopy. In contrast, other cells expressed large amounts of protein-construct and occasionally showed marked abnormalities of cell-morphology, suggestive of cellular decay and death (unpublished data). This phenomenon was previously

observed by others and is considered an artifact of the protein-construct transfection method, as reviewed in [118].

Initially, we attributed these cellular effects to artifacts related to the specific features of the TM6SF2 plasmid-construct used in these experiments and we focused in our reporting on the results of transfected cells with normal cell-morphology. However, in subsequent studies involving plasmid constructs for the overexpression of several different proteins (including PNPLA2 and ABHD5), we again noted effects of the degree of overexpression on cell-morphology. In addition, we observed that "low level" of expression led to the localization of the GFP-tagged proteins in the cytoplasm whereas we did not observe co-localization of ABHD5 protein-construct with lipid-droplets. However, protein expression at "high levels" led to the deposition of ABHD5 protein-construct in various subcellular compartments apart from the cytoplasm, including lipid-droplets as shown in *Figure 16*. This is perhaps not surprising since lipophilic proteins like ABHD5 and PNPLA2 tend to adhere to membranes. In contrast, employing the monoclonal antibody method we did not find any evidence for the co-localization of ABHD5 or PNPLA2 with lipid-droplets. We conclude that overexpression studies can lead to artifacts that can generate "false" co-localizations of lipophilic proteins to the lipid-droplets.

Figure 16. Cell transfection with expression plasmid.

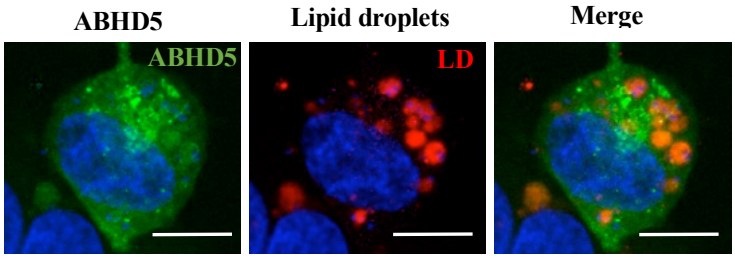

Figure 16. Representative confocal microscopy image of transfected Huh7 cell with GFP-tagged ABHD5 overexpression vector (green). Lipid droplets (red) and nucleus (blue) were stained with Lipid-Tox Red and DAPI respectively. Scale bar, 10 μm.

5.5 ROLE OF TM6SF2 IN HEPATIC TRIGLYCERIDE METABOLISM

It is generally assumed that variation in TRL secretion is the result of changes in the availability of triglycerides for TRL synthesis by the liver [43,44,119]. This hypothesis is supported, among others, by studies that have shown that increased availability of fatty acids to hepatocytes lead to increased cellular triglyceride concentrations and enhanced secretion of TRLs, as reviewed in [48]. Contrary to this generally accepted hypothesis, we observed in Paper I that *TM6SF2* siRNA inhibition of human hepatoma cells leads to *increased* cellular triglyceride content and a *reduced* secretion of TLRs. We noted that these reciprocal changes are in agreement with the population genetics analysis of the *TM6SF2* locus [75,120], but we did not discuss the putative pathophysiological mechanism of this observation.

Modulation of *TM6SF2* expression in mouse model results in similar phenotype compared to the phenotype that we report in Paper I [121–123]. Smagris et al showed that the opposing effects of TM6SF2 on hepatic triglyceride metabolism are due to a role of TM6SF2 in neutral-lipid addition during the synthesis of TRLs [123]. They proposed that a reduction of TM6SF2 diminishes the incorporation of triglycerides in TRLs, thereby leading to accumulation of triglycerides in hepatic lipid-droplets. This hypothesis is compatible with the observed subcellular localization of TM6SF2 in endoplasmic reticulum, as reported in Paper I. However, it is worth mentioning two unpublished observations in human hepatoma cells which are not compatible with this hypothesis. First, it is well known that hepatoma cells exhibit a low secretion rate of relatively triglyceride-poor TRLs [91]. This inhibition of TRL secretion by itself is not sufficient for the generation of a detectable increase in cellular triglyceride concentration. A quantitative analysis of triglyceride secretion by hepatoma cells confirmed that the triglycerides required for TRL secretion are not sufficient to explain the observed increase in cellular triglyceride concentration after *TM6SF2* siRNA inhibition. Secondly, by using *APOB* siRNA inhibition we were able to decrease TRL secretion by >90% in the human hepatoma cell-lines. However, no detectable increase in cellular triglyceride concentration was observed. It is noteworthy that treatment of patients with Familial Hypercholesterolemia with Mipomersen, a second-generation APOB antisense oligonucleotide, is associated with only minor, transient increases in liver fat [124,125]. Taken together, our studies in human hepatoma cells indicate that *TM6SF2* exerts an effect on cellular triglyceride concentration that is not directly bound to TRL secretion. Of note, Ruhanen et al. recently proposed a role of TM6SF2 in altering membrane fatty-acid composition, suggesting that alternative mechanisms may account for the impact of TM6SF2 on TRL secretion and lipid-droplet metabolism [126].

5.6 ROLE OF PNPLA2 IN HEPATIC TRIGLYCERIDE METABOLISM

In order to better understand the triglyceride metabolism in the human liver, we tried to identify the putative triglyceride hydrolase in Paper II by evaluating the role of the three highly expressed members of the PNPLA family in human hepatoma cells. Contrary to the adipose tissue, the hepatic function of the PNPLA family proteins remains unclear. We show that only reduction of the *PNPLA2* hepatic expression, and not of *PNPLA3* and *PNPLA4,* is associated with reduction of triglyceride hydrolysis and of the TRL secretion in our hepatoma model. In contrast, previous *in vitro* and population studies assigned a role of PNPLA3 in hepatic lipid-droplet hydrolysis and VLDL secretion due to its *in vitro* hydrolase activity and due to the phenotype of the I148M polymorphism respectively [127]. However, subsequent study found that the physiological role for PNPLA3 is mainly restricted to mediate the remodeling of triglycerides and phospholipids on lipid-droplets [128].

We found that *PNPLA2* siRNA inhibition resulted in a decrease of the total cellular triglyceride hydrolysis by approximately 50%. This suggests, in agreement with previous studies that PNPLA2 plays a role in hepatic triglyceride hydrolysis [35,129]. However, it was puzzling that we did not observe a greater decrease in cellular triglyceride hydrolysis, considering the near complete reduction of *PNPLA2* RNA concentration and PNPLA2 protein concentration following *PNPLA2* siRNA inhibition. One possible explanation of the phenomenon is that the PNPLA2 protein remaining after *PNPLA2* siRNA inhibition is able to provide approximately 50% of the cellular triglyceride activity in the hepatoma cells. However, we observed a comparable 50% reduction of cellular triglyceride hydrolysis following PNPLA2 inhibition by Atglistatin. Moreover, no additive effects of inhibition with both *PNPLA2* siRNA and Atglistatin were observed. This indicates that a complete inhibition of all PNPLA2-dependent triglyceride hydrolysis accounts for approximately 50% of the overall cellular triglyceride hydrolase activity in human hepatoma cells. This suggests that the remaining 50% of the cellular triglyceride hydrolase activity is provided by alternative, currently unknown triglyceride hydrolases, as proposed by others [35,130].

The subsequently evaluation of the effect of *PNPLA2* inhibition on TRL secretion revealed that both the secretions of triglyceride and APOB decreased following *PNPLA2* siRNA inhibition, PNPLA2 inhibition with Atglistatin and the combined inhibition of PNPLA2 with *PNPLA2* siRNA inhibition and Atglistatin treatment. In contrast, it was reported that adenoviral-mediated *PNPLA2* knockdown and hepatocyte-specific genetic deletion of *PNPLA2* did not influence VLDL secretion [131,132] as reviewed in [35]. We attribute this discrepancy in the secretion of TRLs to species-specific differences in TRL secretion between humans and rodents. Again, it was found that *PNPLA2* siRNA inhibition, PNPLA2 inhibition with Atglistatin and the combined inhibition of PNPLA2 with *PNPLA2* siRNA inhibition and Atglistatin treatment lead to approximately 30% reductions of TRL secretion in human hepatoma cells. This indicates that that there are other mechanisms besides PNPLA2 that regulate the supply of fatty acids for TRL synthesis [35].

Finally, we observed that inhibition of PNPLA2 did not affect the cellular triglyceride concentration or influence the size, number or distribution of the lipid droplets as examined by confocal microscope, despite a 50% reduction of the cellular triglyceride hydrolysis activity of the hepatoma cells. In agreement with this observation, we found that PNPLA2 was not localized around lipid-droplets, suggesting that the triglycerides present in lipid-droplets visualized by confocal microscopy are not the substrate for PNPLA2-mediated triglyceride hydrolysis. In contrast, we found that PNPLA2 was present in the cytoplasm of the hepatoma cells. Moreover, we observed co-localization of PNPLA2 with the ER and PLIN2, indicating that PNPLA2 is associated with small lipid-droplets near the ER. It is in this respect important to mention the presence of different lipid-droplet subpopulations in hepatoma cells: large lipid-droplets (designated L-LDs) that are visible by confocal microscopy, and small lipid-droplets (designated S-LD) that are only visible by electron microscopy [133]. It is likely that these different lipid-droplet subpopulation have different metabolic functions, as discussed below [28].

5.7 ROLE OF ABHD5 IN HEPATIC TRIGLYCERIDE METABOLISM

In Paper III we evaluated the functional role of ABHD5 and its relationship with PNPLA2 regarding cellular triglyceride hydrolysis, TRL secretion, lipid-droplet metabolism and subcellular localization in human hepatoma Huh7 cells. It was found that*ABHD5* siRNA inhibition is associated with reduced cellular triglyceride hydrolysis in Huh7 hepatoma cells. A comparable, partial reduction of cellular triglyceride hydrolysis activity after *ABHD5 siRNA* was previously observed in liver-specific knock-down of *ABHD5* in mice [37]. In agreement with previous studies in murine models [37,39,115], it was found that *ABHD5* siRNA inhibition decreased TRL secretion in Huh7 cells by approximately 50%. However, no effects of *ABHD5* siRNA inhibition on cellular triglyceride concentration or on the concentration and size of the lipid-droplets was observed in confocal microscopy studies. Finally, it was found in subcellular localization studies that ABHD5 was not associated with lipid-droplets, but was present in the cytoplasm of the Huh7 hepatoma cells, near the ER. Overall, our analysis of the role of ABHD5 in triglyceride metabolism in Huh7 cells shows remarkable similarities with the previous analysis (Paper II) of the role of PNPLA2 in triglyceride metabolism of human hepatoma cells. Indeed, a head-to-head comparison of the effects of *ABHD5* and *PNPLA2* siRNA inhibitions on triglyceride metabolism in Huh7 cells found no evidence for distinct differences between the two interventions. Moreover, no additive effects were observed when the *ABHD5* and *PNPLA2* siRNA inhibitions were combined and compared with either *ABHD5* siRNA or *PNPLA2* siRNA inhibitions. These observations suggest that both ABHD5 and PNPLA2 are involved in the same metabolic network related to cellular triglyceride metabolism in human hepatoma cells. However, it must be stressed that in this study we only analyzed the effects of *ABHD5* and *PNPLA2* siRNA inhibitions on specific functions of hepatic triglyceride metabolism, and it remains

therefore possible that ABHD5 exhibits a PNPLA2-independent role in lipid homeostasis, as proposed by Lord et al [134].

The subcellular localization studies reinforced our conclusion that ABHD5 and PNPLA2 act in the same metabolic network in Huh7 cells. We found a substantial co-localization of ABHD5 and PNPLA2 in Huh7 cells near the ER. Of note, in agreement with our previous analysis of PNPLA2 in Huh7 cells (Paper II), we found no evidence for a localization of ABHD5 around lipid-droplets. Further studies are in progress to define in more detail the subcellular localization of these proteins in Huh7 hepatoma cells. Overall, our studies support the notion that ABHD5 acts as a co-activator of PNPLA2 in Huh7 hepatoma cells, in a similar manner as proposed for the interaction of ABHD5 and PNPLA2 in human adipose tissue [36].

5.8 POTENTIAL ROLE OF SMALL LIPID-DROPLETS IN THE REGULATION OF TRL SECRETION

Papers II and III of this Thesis provide evidence that cellular triglyceride hydrolysis constitutes an important mechanism for the regulation of TRL secretion by human hepatoma cells. It was found that both PNPLA2 and ABHD5 serve important roles in this process, presumably acting as the predominant triglyceride-hydrolase enzyme (PNPLA2) and as activator of triglyceride-hydrolase activity (ABHD5). At the same time, confocal microscopy studies provided no evidence for the co-localization of either PNPLA2 or ABHD5 with large lipid-droplets and no evidence was found for the effects of *PNPLA2* and/or *ABHD5* siRNA inhibitions on cellular lipid-droplet size and lipid-droplet number. This raises the critical question as to the subcellular localization of the triglycerides that are hydrolyzed by the combined action of PNPLA2 and ABHD5 activity in the hepatoma cells. This question, in turn, has important consequences for the nature of the metabolic pathways governing fatty acid metabolism of hepatocytes.

Panel A of *Figure 17* presents a schematic overview of the current working-model regarding the fate of fatty acids in hepatocytes. As shown in this Figure, some fatty acids are generated by the hepatocyte itself, a process termed *de novo* fatty acid synthesis. However, an important source of intracellular fatty acids is derived from the uptake of fatty acids present in the circulation in association with plasma albumin. A third source of fatty acids is the lysosomal hydrolysis of triglycerides present in the lipoprotein remnants removed from the circulation by the liver. These fatty acids are used in hepatocytes for three main purposes: firstly, for beta-oxidation of the fatty acids in the mitochondria; secondly, for storage of the fatty acids in the form of triglycerides in lipid-droplets; and thirdly, for incorporation of the fatty acids in the triglycerides of TRLs secreted by the hepatocytes. The fatty acids stored in the triglycerides of the lipid-droplets, in turn, can be mobilized again and can be used for beta-oxidation or the synthesis of TRLs. All of these processes require considerable intracellular movement of fatty acids between different subcellular compartments of the hepatocyte. These

fatty acid transport processes are facilitated by a variety of transport proteins, receptors and other auxiliary proteins. Nevertheless, the toxic nature of fatty acids will drive the hepatocyte to convert fatty acids into temporary storage in the form of triglycerides, again requiring different proteins for lipid-droplet formation and enzymes for triglyceride synthesis and hydrolysis. In short, the intracellular movement of fatty acids is a complex system that requires a large number of cellular proteins that perform and regulate the demand and supply of fatty acids for various metabolic function in hepatocytes.

The Farese-Walther laboratory recently identified two types of intra-cellular lipid-droplets generated by the ER [20]: 1) large-sized lipid-droplets (L-LDs, also designated as eLDs), equipped with the enzymatic machinery to develop into the mature lipid-droplets observed by confocal microscopy, and 2) small-sized lipid-droplets (S-LDs, also designated as iLDs) that lack the ability to develop into mature lipid-droplets and are therefore not visible by confocal microscopy. The function of the S-LDs is as yet unknown. However, it is tempting to speculate that the triglycerides present in the S-LDs is the substrate for PNPLA2 mediated triglyceride hydrolysis. It is possible that PNPLA2 is attached to the S-LD and that activation of triglyceride hydrolase activity by ABHD5 is required to generate the fatty acids necessary for TRL synthesis. An alternative hypothesis is that PNPLA2 is associated with the TRL-synthesis complex and interacts with the S-LD in a similar fashion as Lipoprotein Lipase with circulating TRLs. This possibility is supported by the strong co-localization of PNPLA2 with Protein disulfide isomerase, a well-known ER-marker that is part of the MTTP complex. Clearly, more studies are required to substantiate the hypothesis that S-LDs are an important source of triglyceride-derived fatty acids for TRL-synthesis.

The immediate follow-up question is the origin of the S-LDs used for TRL-synthesis. It was shown by the Farese-Walther laboratory that the S-LDs and L-LDs are generated using essentially the same synthetic machinery, but this does not exclude the possibility that the S-LDs and L-LDs are synthesized at different locations in the ER. Moreover, it is conceivable that the ER-site for TRL synthesis may be quite far removed from the ER-sites for the *de novo* synthesis of S-LDs. Indeed, it is possible that S-LDs are generated at different sites of the ER, for example in conjunction with the uptake of fatty acids in the vicinity of the cell-membrane, the liberation of fatty acids by lysosomal degradation of remnant lipoproteins, or the *de novo* synthesis of fatty acids, and can act as a convenient subcellular ‘shuttle’ of triglycerides from various sites in the ER to the TRL-synthesis complex. It is possible that this form of intra-cellular fatty acid transport via S-LDs exists in parallel with the transport of fatty acids by fatty-acid binding protein.

Figure 17B provides an update of our current working-model describing the fate of fatty acids in hepatocytes. In this revision of Figure *17A* we *removed* the arrow pointing at the transfer of fatty acids from L-LD to the ER-site for TRL synthesis. Moreover, we *reduced* the significance of direct transfer of fatty acids to the ER-site for TRL synthesis. Instead, we *introduced* in *Figure 17B* the concept of triglyceride transport via S-LDs to the ER-site for TRL synthesis.

Figure 17. Schematic figure representing the model of function of PNPLA2 and ABHD5 in triglyceride hydrolysis and secretion.

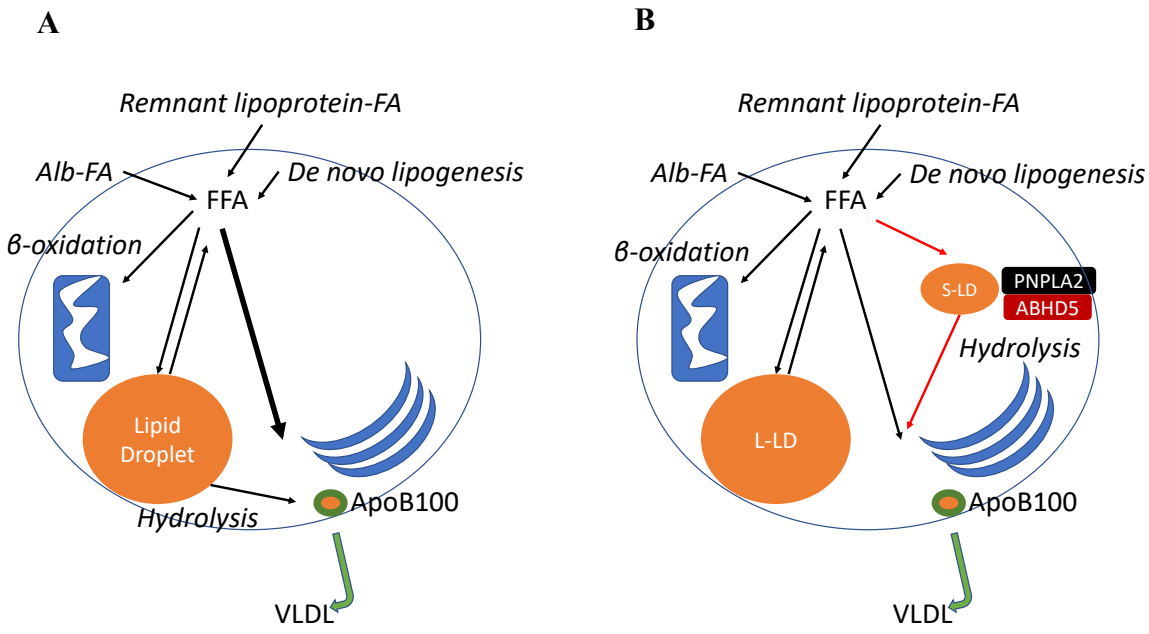

Figure 17. Representative model of triglyceride metabolism in hepatocytes before (A) and after (B) the publication of the present Thesis. The intracellular transport of the fatty acids (FA) is symbolized by the black and red arrows while the secretion of the lipidated apolipoprotein B molecule (APOB100) is represented by the green arrow.

6 CONCLUSIONS

The main findings of this thesis work can be summarized as the following:

1. TM6SF2 is a regulator of liver fat metabolism with opposing effects on the secretion of TRLs and hepatic lipid droplet content.
2. PNPLA2 influences TRL secretion but is not involved in lipid-droplet homeostasis in human hepatoma cells, a physiological role that is quite distinct from the metabolic function of PNPLA2 in non-hepatic tissues.
3. ABHD5 acts as a co-activator of PNPLA2 in Huh7 cells and provide further evidence for a role of ABHD5-PNPLA2 mediated triglyceride hydrolysis in the regulation of TRL secretion.

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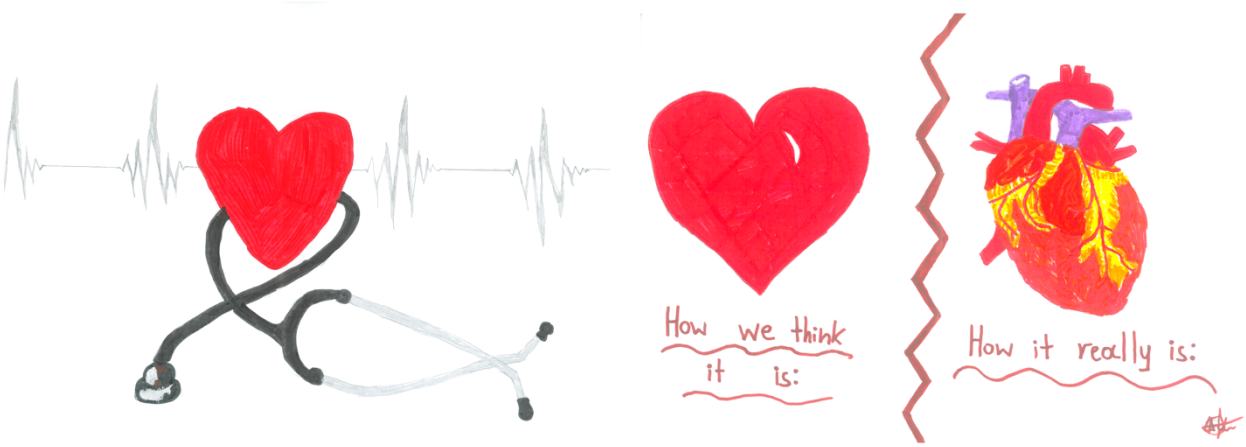

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