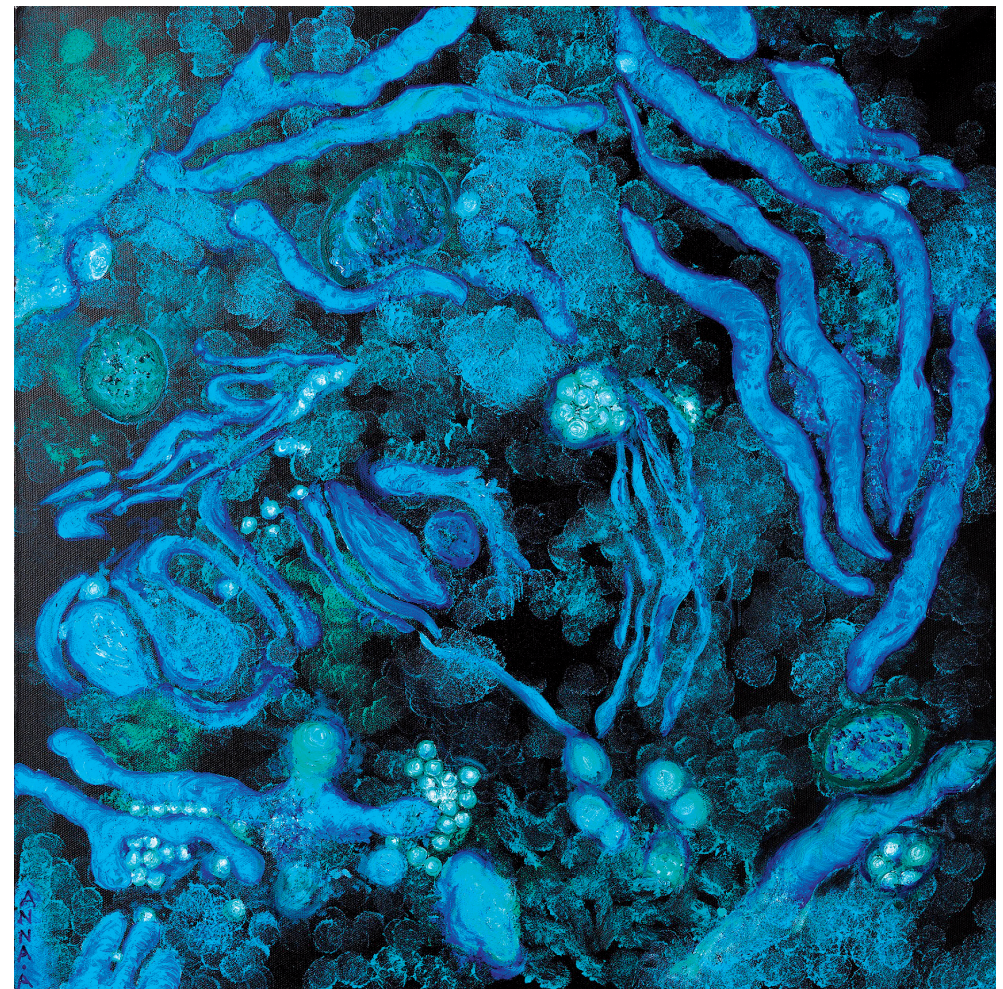


Thesis for doctoral degree (Ph.D.)
2012

Genetic and Functional Studies of MTTP and PLIN2 in Relation to Metabolic and Cardiac Dysfunction



Anna Aminoff

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Lipoprotein assembly

Anna Aminoff, 2012

Oil on canvas

Till Mamma, Pappa, Sofie, Arvid och Alfred

"Our brains appear to be wired for storytelling,
not statistical uncertainty."

Eric Schadt

ABSTRACT

Lipids including triglycerides, cholesterol, cholesterol esters and fatty acids are important sources for energy production, act as building blocks for intracellular compartments, are involved in numerous biological pathways and may act as signal molecules. Lipids are transported in blood as lipoproteins between organs, where they are immediately used in cellular processes or stored as cytosolic lipid droplets. The lipoprotein and intracellular lipid metabolism need to be under tight control to avoid adverse cellular events. Dyslipidaemia and ectopic lipid accumulation are associated with metabolic disorders such as obesity, insulin resistance, type 2 diabetes and a spectrum of cardiovascular diseases.

Microsomal triglyceride transfer protein (MTTP) and perilipin 2 (PLIN2) are two main players in lipid metabolism. MTTP is crucial for the assembly of apolipoprotein B containing lipoproteins, which are mainly secreted by the liver as very low density lipoprotein, and the intestine as chylomicrons. PLIN2 is the main lipid droplet associated protein in non-adipose tissue and is important for the management of intracellular lipid droplets. This thesis investigates genetic variations in *MTTP* and *PLIN2* and their relation to lipid metabolism and metabolic disorders.

A common variant of *MTTP*, comprising two promoter polymorphisms (rs1800591G>T and rs1800804T>C), and a missense polymorphism (rs3816873/Ile128Thr), results in decreased expression of *MTTP* and a less stable protein. The decreased expression, associated with the minor alleles, is mediated by allele-specific binding of nuclear factors to the rs1800804T>C polymorphism. As shown by association studies of cardiovascular diseases, and patients with suspected coronary artery disease undergoing extensively characterisation of their cardiac function, the minor allele of rs1800804T>C confers increased risk for cardiovascular diseases and negatively influences the cardiac function. Decreased cardiac *MTTP* may impair transport of surplus lipids from heart that may cause lipotoxicity and heart failure.

Two patients suffering from Abetalipoproteinaemia were investigated and two novel mutations were identified. Abetalipoproteinaemia is a rare recessive monogenic disease caused by lack-of-function mutations in *MTTP*. The first proband is homozygous for a missense mutation in exon 13 of *MTTP*, p.Pro552Leu (NM_000253.2:c.1655C>T). Amino acid 552 is present in an α -helix domain predicted to bind to protein disulfide isomerase required for functional *MTTP*. There are three other missense mutations reported in exon 13 of *MTTP* that cause Abetalipoproteinaemia. The four missense mutations are associated with different severity of disease, and structural analysis of *MTTP* shows that the position of the mutations may reflect different functional domains of *MTTP*.

The second proband was found to be homozygous for a duplication in the splice junction of intron 17, NM_000253.2:c.2342+2dup. The mother is a heterozygous carrier of this mutation, while no aberrations could be found in *MTTP* of the father. *MTTP* is located at 4q22-24, and analysis of microsatellite markers across the complete chromosome 4 showed that the proband has inherited two copies of chromosome 4 from only the mother, a condition called uniparental disomy. As a result of crossing over events, the interstitial region comprising *MTTP*, is inherited from only one of the mother's chromosome 4, while the telomeric regions originate from both of the two maternal chromosomes. This explains why the proband is homozygous for the mutation while the mother is heterozygous.

Genetic analysis of *PLIN2* identified a missense polymorphism in exon 6, rs35568725 (Ser251Pro). The minor Pro251 allele is associated with decreased plasma triglyceride and very low density lipoprotein concentrations. Functional studies showed that the minor Pro251 allele disrupts an α -helix, is evolutionarily conserved, increases intracellular lipid accumulation and reduces lipolysis. This is the first time a genetic variant of *PLIN2* has been shown to influence the lipid metabolism in humans. The Pro251 variant alters the function of *PLIN2* and results in more stable lipid droplets, and appears to mediate an increased capacity to store intracellular lipids.

The increased understanding of lipid metabolism in the past decade highlights that it is not the amount or concentration of lipid that is the most important issue for maintaining lipid homeostasis. In order to understand the underlying pathophysiology of metabolic disorders we need to address questions related to *where*, *how* and *why* different kinds of lipids are stored and used.

LIST OF PUBLICATIONS

- I. **Aminoff A**, Ledmyr H, Thulin P, Lundell K, Nunez L, Strandhagen E, Murphy C, Lidberg U, Westerbacka J, Franco-Cereceda A, Liska J, Nielsen LB, Gåfvels M, Mannila M N, Hamsten A, Yki-Järvinen H, Thelle D, Eriksson P, Borén J, and Ehrenborg E. Allele-specific regulation of *MTTP* expression influences the risk of ischemic heart disease. *Journal of Lipid Research*. 2009;51:103-111.
- II. **Aminoff A**, Gunnar E, Barbaro M, Mannila M N, Duponchel C, Tosi M, Robinson K L, Hernell O, and Ehrenborg E. Novel mutations in microsomal triglyceride transfer protein including uniparental disomy in two patients with Abetalipoproteinemia. *Clinical Genetics*. 2011. doi: 10.1111/j.1399-0004.2011.01828.x. [Epub ahead of print]
- III. **Aminoff A**, Svedlund S, Mannila M N, Eriksson P, Borén J, Franco-Cereceda A, Gan L-M, and Ehrenborg E. Microsomal triglyceride transfer protein and cardiac function. *Manuscript*.
- IV. **Aminoff A**, Perman J, Mannila M N, Magné J, Neville M, Karpe F, Borén J, and Ehrenborg E. The Pro251 allele in perilipin 2 (*PLIN2*) disrupts an α -helix, affects the lipolysis and is associated with reduced plasma triglyceride concentration. *Manuscript*.

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Kotronen A, Yki-Järvinen H, **Aminoff A**, Bergholm R, Pietiläinen K, Westerbacka J, Talmud P, Humphries S, Hamsten A, Isomaa B, Groop L, Orho-Melander M, Ehrenborg E, and Fisher R. Genetic variation in the ADIPOR2 gene is associated with liver fat content and its surrogate markers in three independent cohorts. *Eur J Endocrinol*. 2009;160:593-602.

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LIST OF ABBREVIATIONS

aa	Amino acid
ABCA1	ATP-binding cassette transporter
ABL	Abetalipoproteinaemia
ACS	Acute coronary syndrome
AMP	Adenosine monophosphate
apo	Apolipoprotein
ATGL	Adipose triglyceride lipase
bp	Base pair
C/EBP	CCAAT/enhancer binding protein
CD1	Cluster of differentiation 1
CE	Cholesterol ester
CETP	Cholesteryl ester transfer protein
CHD	Coronary heart disease
CM	Chylomicron
CVD	Cardiovascular disease
DAG	Diacylglycerol
EMSA	Electromobility shift assay
ER	Endoplasmic reticulum
FA	Fatty acid
GWAS	Genome-wide association study
HDL	High density lipoprotein
HSL	Hormone sensitive lipase
IDL	Intermediate density lipoprotein
IL6	Interleukin 6
LDL	Low density lipoprotein
LDLR	Low density lipoprotein receptor
LPL	Lipoprotein lipase
MAG	Monoacylglycerol
MLPA	Multiplex ligation-dependent amplification
MTTP	Microsomal triglyceride transfer protein
NAFLD	Non-alcoholic fatty liver disease
NASH	Non-alcoholic steatohepatitis
OrO	Red oil O
PDI	Protein disulfide isomerase
PKA	Protein kinase A
PLIN	Perilipin
PPAR	Peroxisome proliferator-activated receptor
sdLDL	Small dense LDL
SRE	Sterol response element
SREBP	Sterol response element binding protein
TG	Triglyceride (triacylglycerol)
TNF	Tumour necrosis factor
UPD	Uniparental disomy

1 INTRODUCTION

The ability to utilise and store neutral lipids is evolutionary conserved and observed in all eukaryotes, reflecting the importance of efficient management of lipids to survival. The uptake, package, storage, transportation and utilisation of lipids needs to be tightly controlled and involves, if not all, numerous cellular functions, and any disturbance of the lipid homeostasis will have physiological implications. The western life style inflicts the lipid homeostasis and results in metabolic disorders, including cardiovascular diseases (CVDs), insulin resistance, type 2 diabetes and obesity. Likewise, there are several genetically derived disorders, i.e. monogenic disorders, of the lipid metabolism with more or less serious health consequences. This thesis focuses on two main players in lipid metabolism, microsomal triglyceride transfer protein (MTTP) and perilipin 2 (PLIN2).

1.1 LIPID METABOLISM IN HEALTH

1.1.1 Lipoprotein metabolism

Lipoprotein particles serve to transport cholesterol esters (CEs) and triglycerides (TGs) in the blood stream (and lymph) to deliver lipids to different organs and tissues. Neutral lipids like TGs and CEs are non-polar molecules that need to be packaged in a soluble form for transportation. The lipoprotein particle has therefore a core of neutral lipids and a surface of more polar lipids, i.e. phospholipids, and proteins, i.e. apolipoproteins (apo) that contain cell-targeting signals. There are several different lipoprotein species including chylomicrons (CMs), CM remnants, very low density lipoproteins (VLDLs), intermediate density lipoproteins (IDLs), low density lipoproteins (LDLs) and high density lipoproteins (HDLs) that vary in size, lipid content and their specific apolipoprotein(s) bound to the surface (See **Figure 1**).¹

Assembly of apoB-containing lipoproteins

Dietary lipids are absorbed by the intestine and incorporated into CMs, which are secreted into the lymphatic system and enter the systemic circulation by the thoracic duct. CM is the most buoyant lipoprotein containing a high fraction of TGs, and is assembled by a two-step process involving MTTP and apoB48, the truncated form of apoB100.² Newly synthesised apoB48 is chaperoned by MTTP in the rough endoplasmic reticulum (ER) and forms a stable complex with phospholipids, cholesterol and small amount of TGs. In the smooth ER MTTP forms a small particle containing TGs, CEs and apoAIV. The two premature CM-particles are fused together and bud from the ER forming a prechylomicron transport vesicle. In the Golgi apoAI is fused to the premature CM generating a mature particle that is secreted from the cell. The secreted CM does not contain MTTP.³⁻⁵

Endogenous lipids, obtained from plasma or *de novo* synthesised, are packaged by the liver forming VLDLs that contains apoB100. Just like the CM-assembly in the intestine, the VLDL formation takes place in the secretory pathway of the cell, where apoB is translated on ribosomes attached to the ER, and channelled into the ER-lumen. In the same way as the assembly of CMs, the N-terminal of apoB interacts with MTTP that transfers lipids upon the growing apoB-chain forming a primordial particle (pre-VLDL). The pre-VLDL is further lipidated generating a TG-poor VLDL-particle (VLDL2) that buds from the ER, and is transported to the Golgi where it may be secreted or acquire additional TGs to form a large TG-rich lipoprotein (VLDL1).⁵⁻⁸

The primordial lipoprotein particles (pre-VLDL and pre-CM) are not secreted from the cells, and will be degraded unless further lipidation occurs. Likewise, the apoB-molecule is sorted for proteasomal degradation if not stabilised by lipids, MTTP and other ER present chaperones.^{6,7} The liver and intestine are the major organs for apoB-lipoprotein assembly and secretion but also the heart,⁹⁻¹¹ placenta,¹² and kidney¹³ secrete apoB-containing lipoproteins. Lipoprotein secretion by the heart constitutes a mechanism by which the heart can get rid of surplus lipids that otherwise may be toxic to the cardiomyocyte.¹⁴⁻¹⁶

Lipolysis of lipoproteins

After secretion the CM requires apoCII, apoCIII and apoE and then interacts with the enzyme lipoprotein lipase (LPL) present in the adipose tissue, skeletal muscles, heart, lung, brain, kidney and macrophages. The apoCII activates LPL which hydrolyses TG into fatty acids (FAs) and glycerol for uptake by the tissue. The remnant particle is poor in TGs and enriched in cholesterol. Similar to CM, the VLDL particle requires apoCII and apoE after secretion, even though apoC and apoE have been detected on nascent VLDL. The VLDL-TGs are hydrolysed by LPL in the same manner as CM-TGs, and the VLDL particle becomes smaller and denser forming IDL, which may undergo further hydrolysis by hepatic lipase and become LDL. High concentration of VLDL1 results in increased formation of small dense LDL (sdLDL). The sdLDL particle has a slower clearance and binds more strongly to proteoglycans in the arterial wall promoting atherosclerosis.^{7, 17-19}

Except LPL there are two other important vascular lipases, hepatic lipase present on enterocytes and endothelial lipase. Hepatic lipase participates in the hepatic handling of CM remnants and in the conversion of IDL to LDL. Endothelial lipase is mainly active on phospholipids in HDL. Other proteins of importance for lipolysis of lipoproteins are; the lipase maturation factor 1 needed for correct folding of the lipases, glycosylphosphatidylinositol-anchored HDL-binding protein 1 expressed on endothelial cells where it binds CMs and LPL, thus anchoring the complex to the cell surface, and the glycoproteins angiopoietin-like proteins 3, 4 and 6 which have inhibiting effects on the lipase activity.^{18, 20}

HDL and exchange of lipids

Most organs and tissues are able to secrete excess of cholesterol through HDL. HDL contains apoAI that is synthesised by the liver and intestine. Two thirds of the HDL particles also contain apoAII, synthesised only by the liver. As compared to the apoB-containing lipoproteins, the HDL particles are mainly lipidated after secretion by the act of ATP-binding cassette transporter (ABCA1). The initial lipidation occurs at the cell surface of the intestine and liver where cholesterol and phospholipids are transferred to the apoA-molecule, but the HDL particle will also obtain lipids from other organs (e.g lipid laden macrophages in the arterial wall), as well as from other lipoprotein species. The transfer of lipids between lipoprotein species is facilitated by two enzymes, cholesteryl ester transfer protein (CETP) that transfers neutral lipids between HDL and apoB-containing lipoproteins, and phospholipid transfer protein that transfers phospholipids from VLDL and CM to HDL. Cholesterol removed from tissues by HDL and ABCA1 is esterified by the HDL-associated enzyme lecithin:cholesterol acyltransferase. The process by which tissues get rid of cholesterol by HDL is called reverse cholesterol transport.²¹

Uptake of Lipoprotein Remnants

Remnant lipoproteins are taken up by the liver through receptor mediated mechanisms, and many of the proteins found on the lipoproteins act as ligands for these receptors. The heparin sulphate proteoglycans are implicated in the uptake of apoB-containing lipoproteins and are abundant in the liver. Complementary to heparin sulphate proteoglycans is the LDL-receptor (LDLR), which mainly assists the internalisation of smaller remnant lipoproteins. Other proteins involved in the uptake of lipoproteins by the liver are scavenger receptor class B type 1, and the LDL receptor related protein 1. The HDL particle is taken up by scavenger receptor class B type 1, and most of the cholesterol is then secreted into the bile, i.e. reverse cholesterol transport.²²

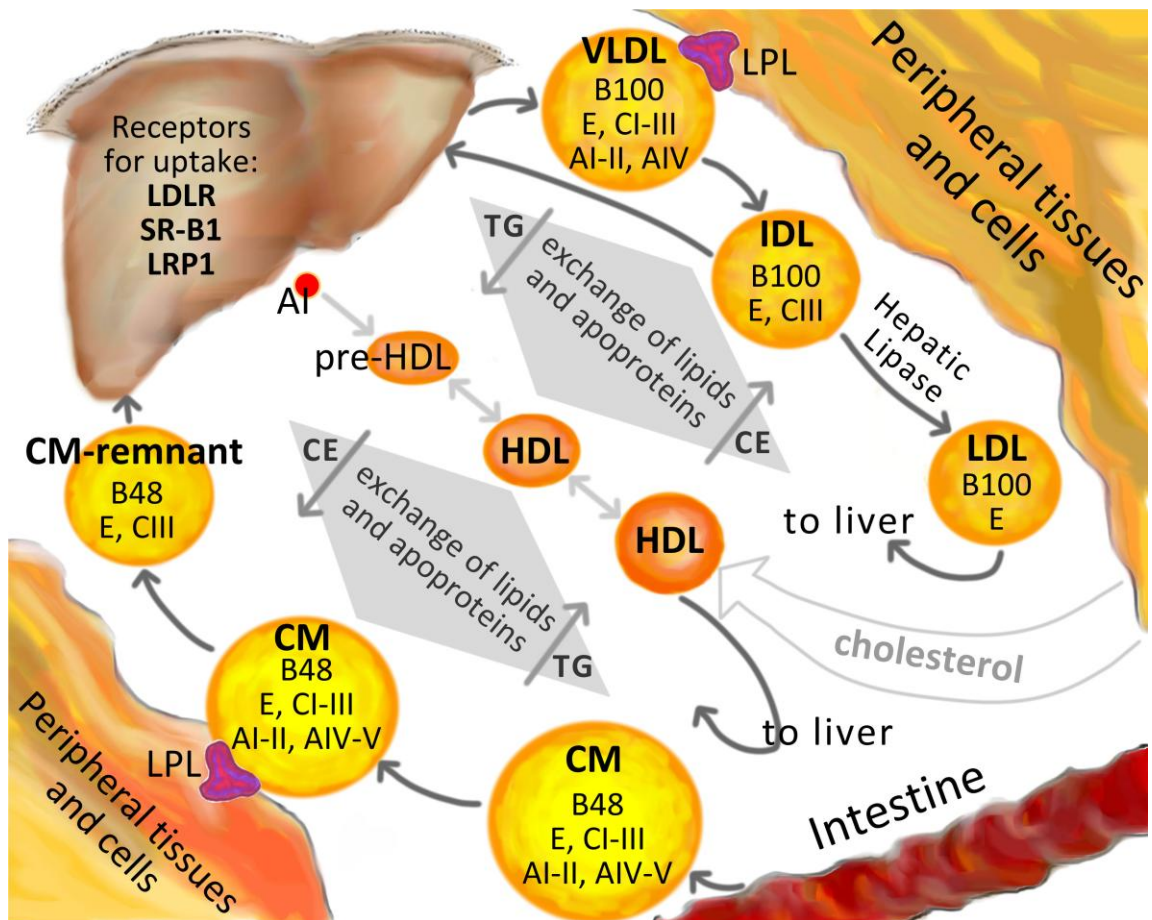


Figure 1. Schematic and simplified overview of lipoprotein metabolism.

Apolipoproteins are referred to as letters (A, B, C and E). There are several different HDL species, which can carry all apolipoproteins except apoB. There is an exchange of apolipoproteins (E, C and A) and lipids between HDL and the apoB-containing lipoproteins. CM, chylomicrons, VLDL, very low density lipoprotein, IDL, intermediate density lipoprotein, LDL, low density lipoprotein, HDL, high density lipoprotein, LPL, lipoprotein lipase, TG, triglyceride, CE, cholesterol ester, LDLR, LDL-receptor, SR-B1, scavenger receptor class B type 1, and LRP1, LDL receptor related protein 1.

1.1.2 Intracellular lipid metabolism

Fatty acid uptake and lipid synthesis

Fatty acids (FAs) are either taken up from plasma or endogenously synthesised by the cell using a large multienzyme complex containing several FA synthetases and the cofactor acyl carrier protein. The uptake of FAs from plasma is facilitated by FA transporter proteins and FA translocase protein (FAT)/CD36. Free FAs are highly toxic to the cell and will be esterified with acyl-CoA by fatty acyl CoA synthase if not immediately used for energy production by β -oxidation. The FA-binding protein family serves many different functions in the FA metabolism by binding FAs and channelling them to different pathways by interaction with other proteins and enzymes. The esterified FAs enter the monoacylglycerol (MAG) pathway to produce diacylglycerol (DAG), which is used for synthesis of TG, phosphatidic choline or phosphatidic ethanolamine. The MAG acyltransferase catalyses the acylation of MAG, forming DAG. DAG can also be synthesised *de novo* by the glycerol-3-phosphate (G3P) pathway by acylation of G3P producing lysophosphatidic acid, which is further acylated and dephosphorylated generating DAG. The DAG is then esterified by acyl CoA:DAG acyltransferase producing TG, the preferable form of lipid for storage.^{23, 24}

Storage of lipids

Neutral lipids, mainly TGs, are stored in the cytosol as lipid droplets. The lipid droplet is a dynamic organelle with a neutral lipid core surrounded by a monolayer of phospholipids and some cholesterol, in addition to attached/integrated proteins involved in the turnover, formation and trafficking of the lipid droplet. The most abundant proteins found on lipid droplets belong to the perilipin family of proteins, consisting of perilipin 1-5. Perilipin 1 and perilipin 4 (also known as S3-12) is mainly expressed in white and brown adipose tissue, whereas perilipin 2 (also known as adipocyte differentiation-related protein, ADRP, ADFP, adipophilin, ADPH) and perilipin 3 (also known as TIP-47) are ubiquitously expressed. Perilipin 5 (also known as OXPAT, LSDA5, LSDP5, and MLDP) is mainly expressed in tissues that have high rates of β -oxidation, such as heart, brown adipose tissue, liver, and skeletal muscle. The formation of lipid droplets takes place at primordial membranes in close proximity to the enzymes involved in the TG synthesis, and the rate by which lipid droplets are formed is dependent on the TG biosynthesis and availability.^{8, 25}

Lipolysis

The lipolysis is tightly regulated to meet the energy demand under different conditions by releasing FAs into blood stream. Three main organs produce and export FAs, white adipose tissue, the intestine and liver, but only the adipose tissue releases “free” fatty acids, i.e. FAs bound to albumin or FA binding protein. The intestine and liver incorporate the FAs in TG and lipoproteins before they are secreted, as described above. Upon catecholamine-induced lipolysis perilipin 1 is phosphorylated by protein kinase A (PKA), adenosine monophosphate (AMP)-activated protein kinase, and mitogen activated protein kinase, supporting docking of hormone sensitive lipase (HSL), which cleaves FA-esters of various compounds. HSL have the highest substrate specificity for DAG. Another important lipase, adipose triglyceride lipase (ATGL) cleaves TG into DAG and FA. ATGL is not hormone sensitive like HSL, even though ATGL is phosphorylated by PKA it is not activated by catecholamines.²⁶⁻²⁸

1.2 LIPID METABOLISM IN DISEASE

Cardiovascular disease (CVD) accounts for approximately 50% of all deaths in Europe and constitutes the main disease burden with an estimated health economical cost of €192 billion.²⁹ CVD is a broad definition generally including coronary heart disease (CHD), heart failure, aortic aneurysm and dissection, peripheral artery disease and stroke. There are several risk factors for CVD, including established atherosclerotic CVD, type 1 and 2 diabetes, family history of CVD, smoking, high blood pressure, male gender, age, and an atherogenic plasma lipid profile, which together can be used to establish the relative future risk of CVD. Individuals at high risk will gain most from preventive efforts, i.e. life-style interventions and medication, and recently it was suggested that special efforts should be focused on improvement of the plasma lipid profile in this group of patients.^{29, 30}

1.2.1 Plasma lipid profile

According to the Medical Products Agency in Sweden an atherogenic plasma lipid profile can be defined by any or several of the following measurements, elevated total cholesterol (> 5.0 mmol/L), elevated LDL-cholesterol (>3.0 mmol/L), elevated TG (>2.0 mmol/L), and low concentration of HDL-C (<1.0 mmol/L). Traditionally, clinical intervention has been focused on managing the overall CVD risk and lowering of the LDL-cholesterol, but despite effective LDL-cholesterol treatment, patients with metabolic abnormalities remain at high risk for CVD. Elevated plasma TG concentration, hypertriglyceridaemia, is now recognised as an independent predictor of CVD, and reflects an increase in TG-rich lipoproteins, i.e. VLDL, CM and their remnants. Metabolic disorders including obesity, the metabolic syndrome, insulin resistance and type 2 diabetes are all associated with dyslipidaemia characterised by high TG and low HDL cholesterol concentrations, predisposing to CVD. The observed

dyslipidaemia is explained by an expanding and/or insulin resistant adipose tissue that sequesters incoming FAs ineffectively, that will result in increased influx of FAs to the liver and an overproduction of VLDL, especially production of TG-rich VLDL particles. Large VLDL particles will compete with CM and its remnants for LPL-mediated catabolism by tissues and receptor-mediated clearance by the liver, prolonging the half-life of the apoB-containing lipoproteins in blood. In plasma CETP subsequently transfers TGs and CEs between lipoprotein species, resulting in atherogenic lipoprotein particles (See **Figure 2**). Moreover, individuals with metabolic disorders also have an increased secretion of apoCIII which further delays the catabolism of VLDL. Large particles cannot diffuse into the vessel wall but small cholesterol-rich lipoproteins readily penetrate the endothelium and binds to the subendothelial matrix initiating atherogenesis. However, large TG-rich lipoproteins have been shown to cause atherosclerosis indirectly by impairing vasodilatation, up-regulating pro-inflammatory cytokine production, enhancing the inflammatory response and activating monocytes, especially in the post-prandial phase when there is an acute elevation of TG-rich lipoprotein remnants.²⁹⁻³⁷

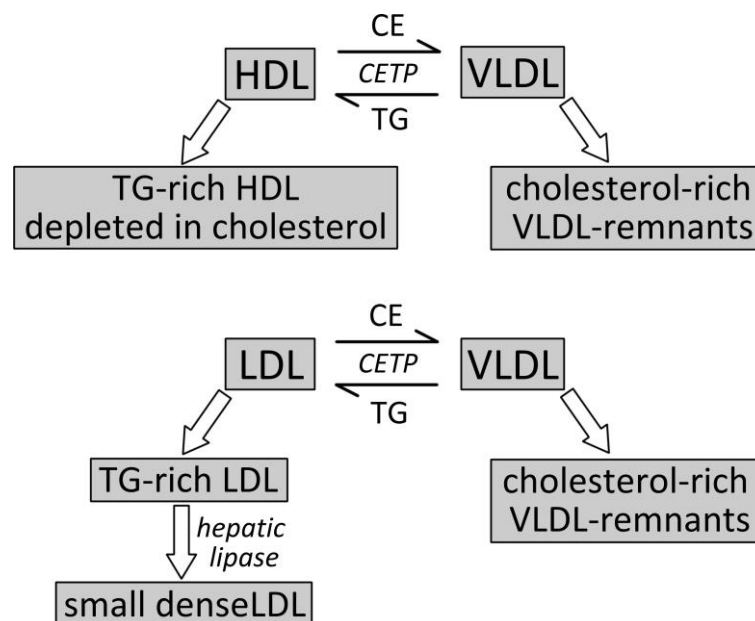


Figure 2. Generation of atherogenic lipoproteins in metabolic disorders. Increased VLDL production results in the production of the highly atherogenic lipoprotein species cholesterol-rich VLDL-remnants and small dense LDL, by the act of cholesterol ester transfer protein (CETP). CE, cholesterol ester, and TG, triglyceride.

1.2.2 Intracellular lipid accumulation

Excess lipid accumulation in non-adipose tissues results in cellular dysfunction, inflammation and eventually cell death, i.e. lipotoxicity, inducing metabolic disorders such as type 2 diabetes and CVD. Lipid accumulation can be facilitated by an increased uptake of lipids, increased synthesis of lipids and/or a decreased oxidation/removal of lipids. When the storage capacity exceeds the oxidation rate there will be an accumulation of toxic lipid intermediates including DAG, ceramides and acyl-CoA with adverse downstream cellular events.^{38, 39} Below follows a description of the pathophysiology of lipid accumulation in vessel wall/ macrophages, liver and heart, which are the main organs/tissues referred to in the four papers the thesis is based on.

Vessel wall and macrophages

Dyslipidaemia causes atherosclerosis as small cholesterol-rich lipoproteins easily enter the vessel wall into the subendothelial matrix where they are modified/oxidised and internalised by macrophages through receptor-mediated endocytosis via CD36 and scavenger receptor A. The internalised cholesterol can be incorporated in membranes and used for cellular processes, channelled to the reverse cholesterol efflux (lipidation of apoAI by ABCA1), or stored in the cytosol as CE in lipid droplets. In metabolic disorders there is a net influx of lipids and a progressive accumulation of cholesterol, transforming the macrophages to lipid-laden macrophages/foam cells. The receptors involved in lipid uptake, CD36 and scavenger receptor A, are not downregulated by intracellular lipids. As more cholesterol accumulates the esterification and storage capacity of CE is exceeded with a concomitant increase in free cholesterol. Free cholesterol is highly toxic as it decreases membrane fluidity, induces apoptosis, causes mitochondrial dysfunction and programmed cell death, activates unfolded protein response, etc. Moreover, free cholesterol will induce macrophage secretion of tumour necrosis factor (TNF) and interleukin 6 (IL6). Modified LDL in the intima will also promote endothelial and smooth muscle cell activation and the expression of adhesion molecules that recruit more inflammatory cells, including T-cells and monocytes. Recruited inflammatory cells will be activated in the intima and enhance the oxidation of LDL, and drive the inflammatory process by production of inflammatory molecules.³⁹⁻⁴¹

Liver

The liver is central for lipid metabolism by its role in lipoprotein metabolism (see above) and ability to produce TG and CE. Even though the liver has a higher capacity to store lipids compared to other non-adipose organs, increased amount of intracellular lipid droplets (>5% of wet weight) cause fatty liver and steatosis. Lipid accumulation in liver may cause non-alcoholic fatty liver disease (NAFLD), which includes everything

from simple steatosis, to non-alcoholic steatohepatitis (NASH), and eventually to cirrhosis. The hallmark of NAFLD is hepatic accumulation of TGs, due to increased influx of FAs and/or *de-novo* lipogenesis. Both obesity and insulin resistance are associated with NAFLD, as these conditions increase lipolysis of TGs in adipose tissue and hence the delivery of albumin-bound FAs to the liver. Insulin resistance also causes hyperinsulinaemia that promotes *de-novo* hepatic TG synthesis, and inhibits β -oxidation of FAs. FAs induce transcription of IL6 and TNF, which increase production of reactive oxygen species, recruit inflammatory cells, and induce apoptosis. TNF also inhibits the insulin sensitive protein adiponectin, which decreases FA export from adipose tissue, inhibits hepatic TG accumulation by reducing FA influx, and increases β -oxidation and lipid secretion.^{38,42}

Heart

The heart consumes more energy than any other organ and has therefore a high demand for oxygen and energy substrates, i.e. glucose and FAs released from lipoproteins by LPL or *free* albumin-bound FAs. FA is an important fuel for cardiomyocytes that generate 60-90% of their energy production from β -oxidation. Most of the FAs that enter the cardiomyocytes are immediately oxidised but some will be incorporated into TG and stored as lipid droplets for later usage. Even though the heart has a high lipid turnover the capacity to store lipids is very limited, and cardiac lipid droplets are generally sparse, small and distributed throughout the sarcoplasm.^{40, 43-45}

Metabolic disorders as obesity, insulin resistance, and type 2 diabetes are all associated with an increased cardiac TG content, partly due to an increased concentration of circulating FAs and an increased deposit.⁴⁶ It has been suggested that cardiac lipid accumulation contributes to the high risk of mortality following myocardial infarction in type 2 diabetics,⁴⁷ and to increase death in mice after acute myocardial infarction are induced.⁴⁸ In animal models increased cardiac lipid content is associated with cardiac dysfunction, apoptosis and heart failure, as well as a higher ceramide content, upregulation of inducible nitric oxide synthase and peroxisome proliferator-activated receptor (PPAR) α regulated genes (TNF and myosin heavy chain β) and downregulation of oxidative pathways.^{38, 47, 49, 50} Reduced production of ATP by downregulation of the oxidative pathways has been suggested to cause decreased contractile function.⁴⁹

New imaging techniques allows a more accurate assessment of both the cardiac lipid content and the cardiac function, and has established that elevated cardiac lipid content in metabolic disorders is associated with impaired cardiac function in humans.^{47, 51-55} Nevertheless, further studies are needed to elucidate how and why cardiac lipid accumulation cause adverse cardiac event in humans.

1.3 TWO MAIN PLAYERS IN LIPID METABOLISM

1.3.1 Microsomal Triglyceride Transfer Protein (MTTP)

MTTP protein and tissue expression

MTTP belongs to a family of lipid transfer proteins that also includes apoB, lipophorin and vitellogenin. MTTP is a large 97kDa protein that has three structural domains; the N-terminal β -barrel that binds to apoB, the middle α -helical region able to associate with both apoB and protein disulfide isomerase (PDI), and the C-terminal β -sheet that mediates the lipid binding and lipid transfer activity of MTTP.⁵⁶⁻⁵⁸ To be fully active MTTP needs to form a heterodimer with the ER-present chaperone PDI (58kD).⁵⁹ The main physiological function of MTTP is to assemble apoB-containing lipoproteins, as described in section 1.1. The tissue expression of MTTP is highest in the liver and in the epithelial cells of the small intestine, the main organs producing apoB-containing lipoproteins, but MTTP is also expressed by other tissues including kidney, heart, retina, neurons, yolk sac, and immunological cells.^{9, 13, 60-62} Both the heart, kidney, and placenta are able to secrete apoB containing lipoproteins (see above).^{10, 13} The expression of MTTP in immunological cells is related to MTTP's role in lipid antigen presentation by cluster of differentiation 1 (CD1) proteins. CD1 molecules are involved in presentation of lipid moieties derived from bacteria, viruses and parasites to T-lymphocytes affecting both the innate and adaptive immune response. MTTP transfer lipids to the CD1 molecules and have been shown to be necessary for the bioactivity of CD1 and the downstream immunological responses of CD1 antigen presentation.^{5, 63}

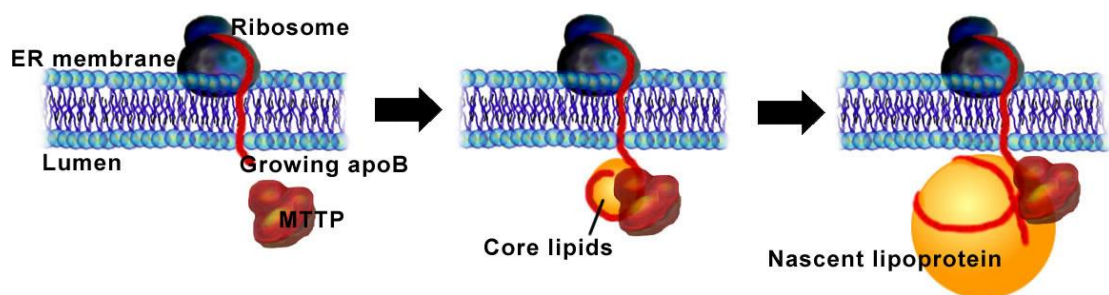


Figure 3. Lipoprotein assembly by MTTP.

Based on figure by Olofsson, S.-O. and Borén, J. *J Intern Med* **258**, 395–410 (2005).⁷

MTTP transcriptional regulation

MTTP is mainly regulated at the transcriptional level, and there is a strong correlation between MTTP mRNA, protein level and activity. The *MTTP* gene is located at 4q22-24, spans approximately 55kb of DNA and consists of 18 exons in humans. Mice have an extra exon located 5' of the gene giving rise to an additional isoform of mttp (*mttp-b*) by alternative splicing of exon 1. In mice *mttp-b* is mainly expressed in cells with low mttp activity, e.g. macrophages, whereas the normal *mttp*, (*mttp-a*) is expressed in liver, intestine and heart.^{64, 65} However, we (unpublished data) and others have not been able to find expression of the MTTP-isoform in humans.^{64, 65} The proximal promoter of *MTTP* contains most of the regulatory elements for *MTTP* transcription summarised in **Table 1**.^{66, 67} *MTTP* is also downregulated indirectly by insulin through the phosphoinositide 3-kinase pathway resulting in reduced binding of FoxA2 and FoxO1 to the *MTTP* promoter, and the mitogen activated protein kinase pathway which results in phosphorylation and translocation of ERK1/2, that is correlated with reduced expression of *MTTP*. ERK1/2 is known to bind to *cis*-element in several genes, but binding of ERK1/2 to the *MTTP* promoter has not been identified.⁶⁷

Table 1. Regulatory elements in the proximal *MTTP* promoter.

<i>cis</i> -element	activator	repressor	co-activator	co-repressor
DR1	RAR/RXR, PPAR/RXR	NR2F1, NR2F2		NCOR1
HNF-4	HNF-4 α			SHP
LRH	LRH-1			SHP
HNF1	HNF-1 α , HNF-1 β			SHP
SRE/IRE		SREBP1, SREBP2		
FOX	FoxA2, FoxO1		PGC-1 α , PGC-1 β	

Table is based on figure by Hussain, M.M., *et al. Clin Lipidol* **6**, 293-303 (2011).⁶⁷

MTTP as drug target

MTTP is crucial for the assembly of CM and VLDL, thus influencing the plasma lipid concentrations of VLDL, CM, and their remnants. New recommendations state that individuals at high risk for CVD would benefit from further reducing their LDL concentration than what is achieved by conventional treatment strategies. Moreover, there are particularly two groups of patients having an unmet clinical need for additional therapies; patients suffering from familial hypercholesterolemia that seldom reach treatment goals, and patients experiencing adverse event from statin treatment. Therefore, new treatment strategies are needed, and MTTP is one of the novel drug targets, currently in phase II and phase III trials. MTTP inhibitors effectively reduce plasma lipid concentration but result in intracellular lipid accumulation. Lomitapide is the most promising MTTP inhibitor which effectively reduces plasma apoB concentrations, and LDL cholesterol with a pronounced effect on the small LDL subfraction. However, treatment with Lomitapide is also associated with elevated liver enzymes, accumulation of hepatic fat and loose stool/diarrhoea.⁶⁸⁻⁷⁰

1.3.2 MTTP deficiency – Abetalipoproteinaemia

Individuals with loss-of-function mutations in MTTP suffer from Abetalipoproteinaemia (ABL), a rare recessive monogenic disorder. ABL patients are unable to produce apoB-containing lipoproteins and have hence very low plasma lipid and apoB concentration, which is often completely absent. Non-functional MTTP results in an inability to absorb dietary lipids and therefore fat soluble vitamin deficiency, as well as intestinal lipid accumulation. The inability to absorb dietary lipids causes diarrhoea which can be alleviated by avoiding fatty food. Also in the liver there is a progressive lipid accumulation causing elevated transaminases, and in some cases the development of liver cirrhosis and carcinoma. The fat soluble vitamin deficiency results in neurological complications due to demyelination of the neurons, and if left untreated may cause mental retardation. The neurological complications include muscle weakness, failure to thrive, spinocerebellar ataxia etc. ABL patients also suffer from retinitis pigmentosa and reduced night vision. Acanthocytosis, abnormal shaped erythrocytes, is another typical ABL-symptom probably caused by deficiencies of iron, folate, and other nutrients secondary to fat malabsorption. The treatment of ABL is diet deviated from lipids, and high dose supplementation of fat soluble vitamin which may reverse the neurological and ophthalmic manifestations.⁷¹

1.3.3 Perilipin 2 (PLIN2)

PLIN2 belongs to the perilipin family of proteins, also known as the PAT-family of proteins, which includes PLIN1-5. (See above for other alias of these proteins.) The family share sequence homology and the ability to bind lipids, and together they manage the intracellular handling of lipid droplets.⁷²

Regulation of PLIN2

PLIN2 is a 48kDa large protein encoded by a 12kb gene located at 9p22.1, containing 9 exons.⁷³ The protein is regulated both at the transcriptional level and post-translationally through ubiquitin-mediated proteasomal degradation. *PLIN2* expression is regulated by PPARs, whose ligands include FAs.^{74, 75} In macrophages phorbol myristate acetate enhances the transcription of *PLIN2* by activating the transcription factors PU.1 and Ap-1 that binds to a Est/Ap-1 element in the promoter.⁷⁶ Unless the PLIN2 protein is stabilised by lipids after translation, it will be targeted for degradation.⁷⁷ Exogenous FAs thus increase PLIN2 levels by both transcriptional activation, and by stabilising the protein through TG and lipid droplet formation. Numerous experiments have shown that increased FA influx is associated with increased *PLIN2* expression and protein levels.⁷²

PLIN2 and lipid droplets in different tissues

PLIN2 is a ubiquitously expressed protein and is the main lipid droplet associated protein in non-adipose tissue, where its expression is highly correlated with the cytosolic pool of neutral lipids.⁷⁸ PLIN2 is also expressed in adipose tissue during adipocyte differentiation where PLIN2 is found on small nascent lipid droplets, but is later replaced by perilipin (PLIN1).^{72, 78}

PLIN2 knockout mice have a very mild phenotype with normal adipogenesis and lipolysis, which could be explained by a compensatory expression of perilipin 3 (PLIN3). However, they do have reduced (~60%) hepatic TG content and are resistant to diet-induced steatosis. Surprisingly, they have a normal rate of VLDL formation and secretion. More detailed analysis of the liver showed that the decreased TG content was attributed to less amount of cytosolic lipid droplets, but there was also a concomitant increase of TG in the microsomal compartments where VLDL is assembled.⁷⁹ This is in agreement with *in vitro* studies showing that increased PLIN2 expression promotes increased storage of TGs in cytosolic lipid droplets but reduces the secretion of TGs in apoB-containing lipoproteins. Similar to the PLIN2^{-/-} mice, knockdown of PLIN2 in these cells reduced the amount of cytosolic lipid droplets but increased the secretion of TG. There was also an increase in β -oxidation of FAs.⁸⁰

Moreover, in leptin-deficient obese mice, knockout of PLIN2 or downregulation with antisense oligonucleotide, reduced hepatic TG content and improved the steatosis, as well as the overall glucose homeostasis and insulin resistance.^{81, 82} PLIN2 expression has also been shown to be correlated with number of enlarged (ballooned) hepatocytes in biopsies from patients with non-alcoholic steatohepatosis.⁸³

It is well documented that lipid loading of macrophages and monocytes increases the expression of PLIN2, and that atherosclerotic lesions have high expression of PLIN2. PLIN2 is hence involved in foam cell formation by promoting storage of CEs and TGs in lipid droplets with a parallel reduction of cholesterol efflux.^{40, 84-86}

In a study of insulin resistance in mice, oleic acid resulted in higher intramyocellular lipid content and higher PLIN2 expression as compared to treatment with palmitic acid. The increased TG content and PLIN2 expression was also associated with better insulin sensitivity.⁸⁷ This is in agreement with other experiments showing that there is a difference between oleic acid and palmitic acid in their relative lipotoxicity.⁸⁸ (See general discussion.) In human muscle, PLIN2 was upregulated in circumstances of improved glucose tolerance.⁸⁹ In another study they did not find any association of expression of muscular PLIN2 (or other perilipins) with insulin sensitivity in humans, but showed in a mice myoblast cell line that FAs increase PLIN2 expression, as well as expression of PLIN1 and 3.⁹⁰ Moreover, increased uptake of FAs led to an increase of the cytosolic pool of lipid droplets and a decrease of the translocation of glucose transporter 4 to the plasma membrane, resulting in insulin resistance. Both the translocation of glucose transporter 4, as well as the fusion of lipid droplets, require synaptosomal-associated protein 23, and in condition with excess FAs, synaptosomal-associated protein 23 seems to be distributed to the interior of the cell favouring lipid storage, thus inflicting the insulin mediated glucose uptake.⁹¹

1.4 GENETIC VARIATION AND LIPID METABOLISM

Genome wide association studies (GWAS) identified several new loci (e.g. *APOA5*) and confirmed previously known regions (e.g. *APOE*) influencing lipid parameters.⁹² Many of the loci associated with CVD, or the genes located in these regions, have unknown function. Four of the loci/genes identified by GWAS are involved in lipid metabolism, including *SORT1*, *LPA*, *LIPA*, *LDLR* and *PCSK9*.⁹³

1.4.1 Study designs

Genetic studies of complex pleiotropic diseases have developed greatly during the last decade. Traditionally, genetic association studies were performed by the candidate-gene approach, based on previous knowledge that a particular gene might be involved in the aetiology of a certain disease. The candidate-gene studies have often failed replication. Technical advancement made it possible to perform GWAS, representing an unbiased approach to genetic association studies, based on the common disease-common variant hypothesis. The many successfully performed GWAS have only been able to explain a small percentage of the heritability and variability of complex traits, and typically identify loci in linkage disequilibrium with the actual causative allele. Nevertheless, in both candidate-gene studies and GWAS, robust and accurate phenotyping is important, as well as replication in independent study samples. Sample size is another important factor, influencing the power and outcome, especially in GWAS where correction for multiple testing is a major issue.^{94, 95}

1.4.2 Previous studies of genetic variation in MTTP

There are no reports of genetic association of *MTTP* with plasma lipids or metabolic disorders from GWAS. There are numerous smaller association studies that include *MTTP* polymorphisms (rs1800591G>T, rs1800804T>C, rs3816873/Ile128Thr) and plasma lipids and/or metabolic disorders with ambiguous results, as summarised in **Tables 2-4**. In a genome-wide scan of longevity in Caucasians living in North America, a genetic marker in *MTTP* was associated with increased life-span.⁹⁶ However, the finding failed replications in four independent European studies.⁹⁷⁻¹⁰⁰

Table 2. Summary of *MTTP* association studies related to plasma lipid parameters and CVD (rs1800591G>T, rs1800804T>C and/or rs3816873).

Study sample	N	Sex	Association with minor allele(s)	Year	Ref
Healthy Caucasians.	184	M	Lower LDL-C.	1998	¹⁰¹
ECTIM-study, MI patients, age matched ctrl.	622 cases 728 ctrl	M	No association with MI, coronary artery stenosis or plasma lipids.	1998	¹⁰²
Framingham Offspring, population based, Caucasians.	1226 M 1284 F	M & F	No association with plasma lipids, lipoprotein subclasses or particle size.	2000	¹⁰³
CHD patients, healthy controls.	103 cases 100 ctrl	M	Increased risk of CHD.	2004	¹⁰⁴
LIPAD-study, peripheral artery disease, sex- & age matched ctrl, Caucasians.	433 cases 433 ctrl	M & F	Higher risk for PAD. Higher C, LDL-C & apoB.	2008	¹⁰⁵
INTERGENE-study, IHD patients, sex- & age matched ctrl.	544 cases 544 ctrl	M & F	Increased risk of IHD.	2010	¹⁰⁶
WOSCOPS, Caucasians CHD patients, healthy ctrl.	580 cases 1160 ctrl	M	Increased risk of CHD. Lower plasma C.	2004	¹⁰⁴
WOSCOPS, Caucasians Healthy individuals.	1117	M	Lower plasma C.	2002	¹⁰⁷
Healthy individuals, French.	326	M & F	Lower plasma TG, C & LDL-C. Increased plasma HDL-C. Lower carotid IMT (N=74).	1998	¹⁰⁸
Healthy individuals, Caucasians.	129	M & F	No association with carotenoids.	2009	¹⁰⁹
Healthy Caucasian males.	564	M	Lower plasma C, LDL-C & LDL-apoB.	2002	¹⁰⁷
Recruit by genotype from above study.	60	M	Increased small apoB48 lipoproteins after oral fat meal. No association with plasma TG.	2002	¹¹⁰
Healthy individuals.	227	M	No association with plasma lipids. Decreased fasting insulin.	2002	¹¹¹
Healthy individuals, Italy.	290	M	Lower LDL-C & resistin.	2007	¹¹²
Moderate CVD risk 3 month diet intervention.	169	M & F	Difference in plasma FA composition & apoB48 concentration.	2007	¹¹³
Metabolic Syndrome (MS) (IDF-criteria), healthy ctrl, Caucasians.	184 ctrl 86 MS	M & F	Higher insulin, NEFA, C-peptide, HOMA-index, TG, VLDL-C & VLDL-TG in males with MS.	2008	¹¹⁴
CARDIA-study, young black men.	586	M	Higher plasma C & LDL-C.	2000	^{115, 116}
KORA-study, population based, Germany.	7582	M & F	No association with BMI, waist or C. (H297Q associated with BMI, waist & C in females.)	2008	¹¹⁷
Young healthy men.	101	M	Increased malondialdehyde-modified LDL & TG, smaller VLDL size.	2009	¹¹⁸
Heterozygous FH patients.	217 M 211 F	M & F	Lower plasma TG. No association with LDL-C.	2000	¹¹⁹
Spanish FH patients.	222	M & F	Lower plasma TG & VLDL in females.	2005	¹²⁰

F, females, M, males, ctrl, control, MI, myocardial infarction, C, cholesterol, CHD, coronary heart disease, CVD, cardiovascular disease, IDF, international diabetes foundation, MS metabolic syndrome, IHD, ischemic heart disease, IMT, intima media thickness.

Table 3. Summary of *MTTP* association studies related to plasma lipid parameters, insulin resistance and type 2 diabetes (rs1800591G>T, rs1800804T>C and/or rs3816873).

Study sample	N	Sex	Association with minor allele(s)	Year	Ref
Patients with T2D.	271	M & F	Lower alanine aminotransferase	2000	¹²¹
Patients with T2D, non-diabetic ctrl, Chinese.	281 T2D 364 ctrl	M & F	No association with diabetes or plasma lipid variables. Higher concentration of sdLDL in diabetic patients.	2003	¹²²
Population based, French-Canadians.	1742	M & F	No association with insulin resistance.	2005	¹²³
MICK-study, healthy individuals.	716	M	Lower postprandial insulin & DBP. Lower prevalence of IGT.	2006	¹²⁴
EPIC-study, T2D patients, sex- & age matched ctrl.	190 T2D 380 ctrl	M & F	Lower incidence of T2D in males.	2006	¹²⁵

F, females, M, males, ctrl, control, T2D, type 2 diabetes, IGT, insulin glucose tolerance.

Table 4. Summary of *MTTP* association study related to steatosis (rs1800591G>T, rs1800804T>C and/or rs3816873).

Study sample	N	Sex	Association with common allele(s)	Year	Ref
NASH patients, healthy ctrl.	63 cases 150 ctrl	M & F	Higher amount of hepatic fat among NASH patients. Common allele more frequent in NASH patients.	2004	¹²⁵
NASH patients, non-obese/non-diabetic healthy ctrl.	29 cases 27 ctrl	M & F	Higher degree of steatosis, more atherogenic postprandial plasma lipid profile.	2007	¹²⁶
Patients with fatty liver, healthy ctrl.	195 cases 393	?	Central obesity, elevated liver enzymes. Common allele more frequent in patients with fatty liver.	2009	¹²⁷
NASH patients, non-obese/non-diabetic healthy ctrl.	40 cases 40 ctrl	M & F	Higher degree of steatosis Among NASH patients. Poorer (OGTT) in both groups.	2010	¹²⁸
NAFLD patients, healthy ctrl.	131 cases 141 ctrl	M & F	No association with NASH.	2010	¹²⁹
NAFLD patients, healthy ctrl.	83 cases 93 ctrl	M & F	No association with NASH.	2011	¹³⁰
Patients with chronic hepatitis C.	86	M & F	No association with hepatitis-Induced steatosis.	2006	¹³¹
Patients with chronic hepatitis C.	102	M & F	<u>Association with minor allele:</u> Higher degree of steatosis in patients with HCV 3 genotype.	2008	¹³²
Patients with chronic hepatitis C.	298	M & F	<u>Association with minor allele:</u> Higher degree of steatosis in patients. Association more pronounced in patients with HCV 3 genotype.	2009	¹³³

F, females, M, males, ctrl, control, NASH, non-alcoholic steatohepatitis, NAFLD, non-alcoholic fatty liver disease.

2 HYPOTHESIS & AIMS

2.1 HYPOTHESIS

Microsomal triglyceride transfer protein (MTTP) and Perilipin 2 (PLIN2) are central players in the intracellular lipid metabolism and lipid biosynthesis. Any constitutive or induced alterations in their expression and protein structure are likely to have an effect on the lipid accumulation and secretion of lipids in different tissues and cell types, and may thus influence the cardiac function and development of metabolic disorders.

2.2 AIMS

The aim of this thesis is to investigate genetic variations and mutations in MTTP and PLIN2, and their consequences for metabolic disorders and lipid metabolism.

2.2.1 Specific Objectives

1. Characterise functional *MTTP* promoter polymorphisms.
2. Study functional genetic variants of *MTTP* in human myocardium in relation to cardiac function in patients with suspected coronary artery disease.
3. Characterise mutations in *MTTP* in patients with Abetalipoproteinaemia.
4. Study *PLIN2* genetic variants and their influence on ectopic lipid accumulation and plasma lipid concentrations.

3 SUBJECTS & METHODS

3.1 SUBJECTS

Below follows a description of the cohorts and subjects investigated within the scope of this thesis. All subjects gave their written informed consent, and the studies have been approved by the local ethical committees by the corresponding universities. Registration numbers of ethical permits are given within [brackets].

3.1.1 The healthy middle-aged men (aka POLCA) (Paper I and IV)

This study consists of 620 healthy 50-year-old men of Northern European descent, randomly selected from a registry comprising permanent residents in Stockholm, Sweden. The cohort was designed for studies on biochemical and molecular genetic mechanisms predisposing to atherosclerosis. Selection of men of identical age was made to eliminate confounding effects of age and gender on lipoprotein metabolism. The participants were extensively characterised with respect to anthropometric, metabolic and inflammatory variables. Exclusion criteria included any physical or mental disorders, and alcohol- or drug related problems.¹³⁴ [96-097; 02-091]

3.1.2 Oxford Biobank (Paper IV)

The Oxford Biobank is a randomised population based cohort of 30- to 50-year-old men and women from Oxfordshire, UK. The cohort was designed for studies on genetic variation in relation to anthropometric and metabolic characteristics. Only healthy individuals were included, and all participants were of white European origin. Exclusion criteria included mental or physical illness, alcohol- or drug-related problems, and abnormal biochemical data as determined by history, examination, routine blood tests or information obtained from each subject's primary care physician. At the time of analysis, DNA, anthropometric measurements, and plasma lipid profile were available from 1493 individuals.¹³⁵ [08/H0606/107]

3.1.3 INTERGENE Case-Control Cohort (Paper I)

The case-control study is part of the INTERGENE study and includes 544 validated ischemic heart disease patients and 544 age- and sex-matched control subjects living in the Västra Götaland region of Sweden. INTERGENE is a cohort designed to study the interplay between genetic susceptibility, environmental factors, lifestyle, gender, and established risk factors for CVD. The patients are survivors of acute coronary syndrome (ACS) and were identified from the source population (INTERGENE).

There was no lower age limit for the first event and the upper age limit was 75. ACS comprises both acute myocardial infarction (International Classification of Diseases, ICD 10: I21.0-I21.9) and unstable angina pectoris (ICD 10: I20.0). The cases included both previously-known coronary patients with a new episode of ACS, and patients presenting with first time ACS. Full description of the study is available at www.sahlgrenska.gu.se/intergene. [Ö 237-00]

3.1.4 The CEVENT Study

(Paper III)

The study comprises 468 patients with clinically suspected coronary artery disease referred to Department of Clinical Physiology at Sahlgrenska University Hospital for examination of chest pain. All patients underwent echocardiography and myocardial perfusion scintigraphy allowing for extensive characterisation of cardiac performance. Special interest was directed to presence and extent of myocardial ischemia in this mid to high risk patient group. The study was conducted during 2006-2008. [449-06]

3.1.5 Advanced Study of Aortic Pathology, ASAP

(Paper I and IV)

The Advanced Study of Aortic Pathology (ASAP) is an ongoing prospective and observational cohort study of patients undergoing elective open heart surgery for aortic valve or ascending aortic disease. The study was designed to investigate the development and underlying causes, including genetic determinants, for valve disease (aortic stenosis and regurgitation), and ascending aorta dilation (aneurysm or ectasia). Patients aged 18-year-old or above with aortic valve disease and/or ascending aortic disease were included. Exclusion criteria were coronary artery disease and Marfan Syndrome.¹³⁶ DNA was genotyped by Illumina Human 610K chip as described.¹³⁷ [2006/748/-31/1]

Subgroups of the ASAP study were used for analysis of myocardial gene expression with special interest directed towards *MTTP* expression, as described below.

Biopsies from the human heart

(Paper I)

Patients from the ASAP study were selected based on the following criteria; tricuspid aortic valve, Caucasian origin, no type 2 diabetes, and successful RNA isolation from left ventricle biopsy. The selected patients were genotyped for three *MTTP* polymorphisms, rs1800591G>T, rs1800804T>C, and rs3816873 (Ile128Thr). The samples were used for Pyrosequencing analysis which requires heterozygous material, and therefore only patients heterozygous for the *MTTP* polymorphisms were selected (n=9).

Fine needle biopsies from left ventricle of the heart were taken during surgery from 126 individuals and global gene expression were measured by the use of Affymetrix ST 1.0 as described.¹³⁸

3.1.6 Liver biopsies from individuals with steatosis

(Paper I)

A total of 25 Caucasian individuals were recruited from patients undergoing laparoscopic gastric bypass or patients referred to Gastroenterology Department because of impaired liver function. The patients included in the study were between 18- and 60-years-old, consumed less than 20g ethanol per day and none of the subjects suffered from type 2 diabetes, but showed clinical signs of various degree of liver steatosis, i.e. NAFLD, diagnosed by histopathological assessment. Exclusion criteria were chronic hepatitis B or C, NASH, thyroid dysfunction, autoimmune hepatitis, primary biliary cirrhosis, primary sclerosing cholangitis, and use of hepatotoxic drugs or herbal products. A needle or wedge biopsy was taken after an overnight fast. Of the 25 patients six were excluded because of NASH, undefined hepatitis, cirrhosis, or insufficient sample material. Total RNA was isolated and cDNA generated using standard protocols¹³⁹. The patients were genotyped for three *MTTP* polymorphisms rs1800591G>T, rs1800804T>C, and rs3816873 (Ile128Thr), and samples heterozygous for the *MTTP* polymorphisms (*N*=12) were used for Pyrosequencing experiments. [2005/446-31/4]

3.1.7 Primary monocytes from healthy individuals

(Paper I and IV)

A total of sixteen healthy individuals with known genotypes for the *MTTP* rs1800591G>T, rs1800804T>C, and rs3816873 (Ile128Thr) polymorphisms were randomly selected and recruited among Caucasian participants from the POLCA population (Paragraph 3.1.1.). Of these six individuals were heterozygous for the *MTTP* polymorphisms, six were homozygous for the major alleles and four were homozygous for the minor alleles (Paper I). For the studies conducted in paper IV three individuals homozygous for the minor allele of the rs35568725 (Ser251Pro) polymorphism were recruited from the POLCA population, in addition to four controls homozygous for the Ser251-variant. After an overnight fast approximately 100 ml blood was drawn into EDTA tubes, and monocytes were immediately isolated. [96-097; 02-091]

3.1.8 Case Reports: Abetalipoproteinaemia (ABL)

(Paper II)

In paper II two patients suffering from ABL were characterised. Below follows the complete clinical case reports, which were not included in the paper. [2011/870-31/3]

Proband 1

Proband 1 was diagnosed with ABL when he was 15-years-old. The proband was referred to the Department of Paediatrics at the age of 11 years due to short stature. He had also suffered from failure to thrive and aversion to fatty food since his first year. Examination, including capsule biopsy of small intestine, excluded endocrine disorder and celiac disease. He had slightly elevated transaminases; aspartate aminotransferase 1.34 μ kat/L, and alanine aminotransferase 1.36 μ kat/L. Of note, both his parents were of short stature, the mother being 158 cm and the father 160 cm. A dietary history revealed that he had very special food preferences. The baby clinic encouraged the parents to give him energy dense food, as milk with extra cream, but he refused to eat fatty food and preferred vegetables, fruits and fish. He also had a daily consumption of twenty pieces of crisp bread with low fat ham. He did not suffer from diarrhoea, vomits or felt sick to his stomach, but had loose stool quite often.

When the proband was 15-years-old he was referred the second time to the Department of Paediatrics because of his short stature and suspicion of Crohn's disease. The proband had relatively mild ABL-symptoms, i.e. loss of deep tendon reflexes, mild balance disturbance, tongue fasciculations and impaired stereognosis. Upper endoscopy revealed a greyish-white mucosal surface in the duodenum, and examination of the intestinal biopsies (including capsule biopsy) was judged compatible with ABL. Lower endoscopy and barium follow through were normal. Plasma lipid concentrations were low; cholesterol 0.8-1.5 mmol/L, with 75% in the HDL fraction; TG 0-0.03 mmol/L; VLDL-TG 0 mmol/L; apoB not detected; and apoAI and AII below normal levels. Serum iron concentration was low and he had acanthocytosis but no anaemia. At 15 years and 3 months his bone age was estimated to 13 years, and pubertal stage was II according to the Tanner scale. The blood pressure was normal (110/80 mmHg). His energy intake was estimated to 50% of the recommended intake for his age.

Ophthalmologic examination revealed pigmented retina, field of vision showed scotoma, and he had reduced dark vision and colour vision. Electrophysiology showed tapetoretinal degeneration. Somatosensory and visual evoked responses were normal. Electromyography showed axonal polyneuropathy. A pedigree revealed consanguinity 8 generations back.

Following diagnosis, the proband was recommended a low fat diet, and treatment with vitamin E (100 mg/kg/day) was initiated. Analysis of FA composition in erythrocyte membranes and adipose tissue revealed essential FA deficiency, and he was treated with per oral supplements of n-3 long-chain polyunsaturated FAs as fish oil cap-

sules, and capsules with n-6 essential FAs to the level he tolerated without symptoms. He was also given intravenous fat emulsion (Intralipid) on regular basis but that could be discontinued during the first year of treatment. After some time he was also put on extra vitamin A (50 000 IE/day) and K (10 mg/day) due to low serum levels of retinol and prothrombin complex.

He was now followed at the Department of Paediatrics on a regular basis with clinical, laboratory, ophthalmologic and electrophysiological check-ups. His essential FA status and serum concentrations of fat soluble vitamins were normalised, with the exception of vitamin E that remained low. Eleven years after diagnosis his visual impairment was improved with normal dark vision and reduced scotoma. His electroretinogram was improved with normal response from the rods, but still impaired from the cones. Visual and somatosensory evoked responses were normal. Electromyography showed only mild impairment, no longer justifying a diagnosis of polyneuropathy.

Proband 2

Proband 2 was referred to the Department of Paediatrics when he was 2-months-old, due to failure to thrive and low weight, cascade vomits and diarrhoea. Investigation, including explorative laparotomy, excluded pyloric stenosis, common metabolic diseases, and neurologic abnormality. After several admittances because of continued failure to thrive, faecal fat was assessed for suspicion of malabsorption, which revealed increased fat excretion. Enzyme analyses of duodenal contents excluded exocrine pancreatic insufficiency. Investigation of a small intestinal biopsy showed pathology compatible with ABL. Plasma lipid concentrations were low; cholesterol 1.5 mmol/L; TG 0.1 mmol/L; serum apoB and vitamin E were undetectable. A blood smear showed acanthocytosis. Alanine- and aspartate aminotransferases were barely elevated. He had normal psychomotor development and physical examination was normal except a large, protruding abdomen. He was prescribed an infant formula based on extensively hydrolysed proteins and medium-chain TGs, and regained normal weight for his age.

After referral to the Department of Paediatrics at 11 months of age, further investigation revealed normal neuropaediatric clinical examination and normal ophthalmologic examination with no visible retinal pigmentation. Somatosensory and visual evoked responses were normal. His plasma essential FA concentration was low, notably within the n-3 series. Plasma lipid concentrations were also low; cholesterol 0.6 mmol/L; HDL-cholesterol 0.5 mmol/L (83% of normal), TG 0.2 mmol/L, VLDL/LDL undetectable, and apoB 0.04 g/L (0.54-1.67g/L). His serum vitamin concentrations were as follows; vitamin E (α -tocopherol) 1.7 mmol/L (13.5-36.8 mmol/L); vitamin A (retinol) 1.53 mmol/L (0.95-3.31 mmol/L); which successively decreased to 0.56 mmol/L before he was supplemented; and S-25 OH vitamin D normal. Otherwise, his only symptoms were loose stools three times per day.

He was recommended low fat diet but with high relative concentrations of essential FAs, i.e. linoleic- and α -linolenic acid, vitamin E 100 mg/kg/day, and regular supplementation of vitamins A (400 μ g/day) and D (10 μ g/day), at the time recommended as a daily supplement to all children until 5 years of age. At 4 years of age the vitamin E dose was increased to 200 mg/kg/day and vitamin A to 1200 μ g/day and vitamin D to 30 μ g/day. From 7 years of age, oils rich in n-6 and n-3 essential FAs were given as daily supplements and from 8 years he needed supplement with K-vitamin (10 mg/day).

He had clinical, chemical, ophthalmologic and neurophysiologic check-ups every to every second year. At the age of 19-years-old his physical status was normal and so was his ophthalmologic status with normal acuity, no pigmentation, normal field of vision (Goldman) and dark adaptation. Electroretinogram was normal but rod response was slightly reduced compared to examination three years earlier. Somatosensory and visual evoked responses were also within the normal range. S- α -tocopherol was 5 μ mol/L (14-37); s-retinol 1.8 μ mol/L (1.0-3.3 μ mol/L); 25 OH vitamin D 21 nmol/L (25-125); 1,25 (OH)₂ vitamin D 39 nmol/L (10-60 nmol/L); cholesterol<1.29 mmol/L; TG<0.11 mmol/L; HDL-cholesterol 0.56 mmol/L (43% of normal); LDL-cholesterol 0.7 mmol/L; alanine aminotransferase 1.88 μ kat/L (normal range <0.75 μ kat/L); and aspartate aminotransferase 1.49 μ kat/L (normal range <1.1 μ kat/L).

3.2 DNA, cDNA & RNA PROCESSING

This thesis is based on genetic studies, and the experimental procedures related to DNA, cDNA or RNA are hence fundamental. Several of the described experiments uses isolated genomic DNA, which was either isolated by the same laboratory technician, Karin Husman, who uses QIAGEN Blood and Cell Culture DNA kit (QIAGEN Ltd, Crawly, UK) and RapidPrep Macro Genomic DNA Isolation Kit (Pharmacia Biotech, Sweden), or by automatic DNA extraction robots. Below follows a description of the other genetic experiments applied within the scope of this thesis.

3.2.1 Polymerase Chain Reaction, PCR

(Paper I, II and IV)

The polymerase chain reactions (PCR) were performed with polymerase and PCR kit from either Fermentas (St. Leon-Rot, Germany) or Invitrogen (Pailsey, Scotland, UK). Except for being sensitive to the specific buffer of the polymerase, the reactions otherwise contained the same material including 10-200 ng/ μ L DNA, $MgCl_2$, forward and reverse primer, and dNTPs in a total volume between 10-50 μ L. Polymerase and enzyme buffer were added according to manufacturers' advice. Reactions were optimized with regards to annealing temperature, and amplified for 30-50 PCR cycles. Amplification and quality of PCR products were verified by agarose gel separation.

Long-range PCR

(Paper II)

Whereas ordinary PCR-polymerases are efficient in amplifying sequences of a few kilo bases, long-range PCR can amplify fragment up to ten times longer. With increasing length of the amplified fragment dissociation of the polymerase is more likely to occur and result in misincorporation. To overcome this problem, long-range PCR uses a mixture of polymerase and proof reading enzyme. The method requires extensive optimisation of the requisite of the PCR reaction.¹⁴⁰ Long-range PCR was used in paper II in order to detect putative deletions in *MTTP* in patients suffering from ABL and their first degree relatives.

3.2.2 Sequencing

(Paper I, II and IV)

Sequencing was performed by Sanger sequencing methodology based on PCR amplification with addition of dideoxy-nucleotides (ddNTP) that terminates the elongation. Only one of the template strands is amplified. The ddNTPs will be randomly incorporated and terminate the elongation at each position in the targeted sequence, generating fragments corresponding to each position. The ddNTPs are fluorescently labelled with a dye specific for each nucleotide (A, C, T, G). The fragments are separated by an automatic capillary sequencer detecting the fluorescent dyes corresponding to the specific nucleotide at a given position (fragment of certain length).

3.2.3 RNA isolation and cDNA synthesis

(Paper I, II and IV)

RNA was isolated using the RNeasy system (Qiagen, Maryland, USA) and reversed transcribed using poly-dT oligonucleotides and reverse transcriptase, i.e. Superscript II (Invitrogen, Paisley, Scotland, UK), according to manufacturers' advice.

3.2.4 TaqMan genotyping and gene expression

Genotyping

(Paper I-IV)

TaqMan genotyping is a PCR-based assay using fluorescently labelled probes binding to the polymorphic region of interest. The probes are complementary to either one of the two alleles, and upon hybridisation and amplification the polymerase degrades the probe and releases the fluorescent dye by exonuclease activity, generating a light detected by the instrument. DNA homozygous for the major allele only generates signals from the dye specific for the probe binding to the major allele sequence, and vice versa. Heterozygous DNA generates light containing both dyes. By plotting the intensity of the two dyes in a scatter chart, the different genotypes will appear as clusters. TaqMan genotyping was performed in 96-well plates in a total volume of 15µL with 50ng of DNA, or in 384-well plates with dried DNA according to manufacturer's instructions. Genotyping with 384-well plates generates more reliable results and consumes less material.

Gene expression, quantitative real-time PCR

(Paper I, II and IV)

TaqMan gene expression analysis is a quantitative real-time PCR based on the same methodology as TaqMan genotyping. The assay uses a fluorescently labelled probe complementary to a fragment of the coding sequence of the gene of interest, and unlabelled primers. RNA is reversed transcribed and used as template. During amplification the polymerase degrades the probe and releases the fluorescent dye which is detected by the instrument in real time. The amplification of the RNA/cDNA reaches an exponential phase, i.e. the threshold cycle, from which the amount of starting material can be estimated by using internal standards; housekeeping genes and standard curves.^{141, 142}

3.2.5 Restriction Fragment Length Polymorphism, RFLP (Paper II)

Restriction fragment length polymorphism (RFLP) was used for genotyping when other methods, i.e. TaqMan genotyping, were inappropriate for the specific DNA sequence. RFLP genotyping uses restriction enzyme recognising the target sequence including the polymorphic site. Following amplification of the target sequence the amplicons are incubated with restriction enzyme, which only binds and cleaves one of the two alleles/sequences. The DNA fragments are then separated using gel electrophoresis and genotypes are determined based on number and length of the fragments.

3.2.6 Pyrosequencing (Paper I)

Pyrosequencing is a quantitative sequence technology with a wide range of applications. Prior to the Pyrosequencing reaction, DNA or cDNA is amplified using PCR with one of the primers being biotinylated. The PCR amplicon is mixed with sepharose beads, which bind to the biotin molecules. Using a vacuum tool, the sepharose beads with the attached biotinylated PCR product, is denatured with NaOH, which breaks the hydrogen bonds making the DNA single stranded. The single stranded PCR amplicons, attached to the vacuum tool, are then transferred to the Pyrosequencing plate and incubated with a sequencing primer, DNA polymerase, ATP sulfuryase, luciferase, apyrase and the substrates adenosine 5' phosphosulfate and luciferin. The sequencing reaction starts by adding one dNTP at a time in the same order as the nucleotides appear in the target sequence. When the dNTP is incorporated into the DNA strand, pyrophosphate is released in a quantity equal to the amount of incorporated nucleotide, and converted to ATP by ATP sulfuryase and adenosine 5' phosphosulfate. The released ATP drives the luciferase reaction, converting luciferin to oxyluciferin, which generates light that is detected by the instrument and is proportional to the number of nucleotides incorporated. The amount of nucleotide is visualised as peaks in a histogram. Apyrase is needed to degrade unincorporated nucleotides and ATP. Only when the degradation is complete is the next nucleotide added.¹⁴³

The Pyrosequencing technology is highly sensitive and quantitative. In paper I Pyrosequencing was used to detect the relative expression of *MTTP* from each chromosome in material heterozygous for the three *MTTP* polymorphisms rs1800591G>T (-493G>T), rs1800804T>C (-164T>C), and rs3816873 (Ile128Thr). The three polymorphisms are in complete allelic association and therefore can the rs3816873 polymorphism be used as a surrogate marker for the promoter activity mediated by the different alleles of rs1800591G>T and rs1800804T>C. cDNA was used as template to measure the expression level.

To detect any differences in the efficiency by which the different nucleotides are incorporated the assay is normalised to a standard curve. The standard curve was made from pooled PCR products amplified from cDNA from ten individuals homozygous for either the major or the minor alleles of the rs1800591G>T, rs1800804T>C, and rs3816873 (Ile128Thr) polymorphisms respectively, and then mixed in different ratios as follows; 0:10; 1:9; 2:8; 3:7; 4:6; 5:5; 6:4; 7:3; 8:2; 9:1; 10:0.

3.2.7 Multiplex Ligation-Dependent Amplification, MLPA (Paper II)

Multiplex ligation-dependent amplification (MLPA) is a multiplex PCR using multiple oligonucleotides in the same reaction to detect deletions in the targeted DNA. The method is highly sensitive and can distinguish between sequences that differ in only one nucleotide. The method can be used for analysis of copy number variation, including deletions, mRNA profiling and methylation pattern. This section describes the MLPA application related to copy number variation. Oligonucleotides/probes, complementary to the sequences of interest, are designed so that every final probe have a specific length ranging from 84 – 132 bp with 4 bp intervals, does not cover any polymorphic site, is absolutely unique to the targeted sequence, have a GC ratio of ~50%, and does not contain any secondary structure. Optimal probe design will highly benefit the quality of the result. Every probe is composed of two parts, which after the hybridisation to the genomic DNA are ligated. The oligonucleotide pair hybridises to immediately adjacent sequences, and only when both probes are hybridised can they be ligated and later amplified. For ligation purposes the joining base pair must be either G or C. The oligonucleotides are flanked by two universal primer sequences; one derived from phage M13 the other one synthetic, which enables each probe to be amplified by a universal primer pair.¹⁴⁴

The reaction starts with denaturation of genomic DNA whereupon the probes, flanked by the universal primer sequences, are added and hybridised to the DNA by slowly lowering the temperature from 95°C to 60°C followed by incubation for 16-18h at 60°C. All probes have a melting temperature of >65°C. Each pair of half probes is ligated by adding a ligase. The ligated probes are then amplified in an ordinary PCR reaction with 35 cycles with primers complementary to the phage M13-sequence or the synthetic sequence. One of the primers is fluorescently labelled. Noteworthy, the MLPA procedure results in amplification of the probes and not the targeted DNA.^{144, 145}

The amplified fluorescently labelled probes of different length are separated by fragment analysis. The separation is done by a capillary sequencer with fragment software. In the current study the ABI-3730 DNA Analyzer 96 capillaries were used.

3.2.8 Microsatellite analysis

(Paper II)

A microsatellite or a short tandem repeat consists of two or more bases that are tandemly repeated. There are over 100 000 microsatellite loci in the human genome and they often have a high level of heterozygosity, owing to the large variation in the number of repeats.¹⁴⁶ Analysis of microsatellites therefore enables discrimination of paternal and maternal origin of alleles. Microsatellite analysis was used to establish the origin of chromosome 4 in a proband suffering from ABL. Microsatellite markers on chromosome 4 (D4S412, D4S2935, D4S403, D4S419, D4S391, D4S405, D4S1592, D4S392, D4S2964, D4S1534, D4S414, D4S1572, D4S406, D4S402, D4S1575, D4S424, D4S413, D4S1597, D4S1539, D4S415, D4S1535, D4S426) were amplified from 60 ng of genomic DNA by PCR using the Linkage Mapping Set, version 2.5, according to manufacturer's advice (Applied Biosystems, Foster City, CA, USA). Amplified microsatellites were separated using gel separation (ABI genetic analyser 3130, Applied Biosystems), and alleles were detected using the software GeneMapper 4.1 (Applied Biosystems, Foster City, CA, USA).

3.3 CELL CULTURE EXPERIMENTS

3.3.1 Isolation and differentiation of monocytes

(Paper I and IV)

In paper I and IV human peripheral blood mononuclear cells were isolated using Ficoll-Paque according to the manufacturer's instructions (GE Healthcare Bio-Sciences, Uppsala, Sweden). The isolated primary cells were used for differentiation into macrophages by phorbol myristate acetate treatment as described elsewhere.¹⁴⁷ To allow complete differentiation of monocytes, cells were cultured for another 60 h at 37°C. The macrophages were cultured under basal conditions.

3.3.2 Electromobility Shift Assay, EMSA

(Paper I)

Electromobility shift assay (EMSA) is based on the rationale that molecules with different size, molecular weight and charge have diverse electrophoretic mobilities, and will hence be separated in a polyacrylamide gel. In paper I, EMSAs were used to detect allele-specific binding of sterol response element binding protein (SREBP) and CCAAT/enhancer binding protein (C/EBP) to the rs1800804T>C polymorphic site in the *MTTP* promoter. Oligonucleotides encompassing the polymorphic site, C/EBP consensus and a sterol response element (SRE) were radioactively labelled and incubated with either *in vitro* translated protein or nuclear extract from liver cell lines, and loaded onto the gel and separated. To visualise specific binding of C/EBP to the rs1800804T>C polymorphic site, competition with unlabelled

C/EBP consensus oligonucleotides were used. Putative binding of SREBP to the polymorphic site was investigated by adding SREBP-antibody. DNA bound to a protein, or a larger protein i.e. antibody, will migrate more slowly compared with free DNA and cause a shift. The bands are visualised by drying the gel and placing it on a screen detecting radioactivity.¹⁴⁸

3.3.3 Minigen plasmid experiments

(Paper II)

In paper II a functional splicing minigene assay was used to evaluate a duplication (NM_000253.2:c.2342+2dup) assumed to cause skipping of exon 17 in *MTTP*, and hence ABL. The minigene assay relies on an expression vector pcDNA3.1, containing the cytomegalovirus promoter and two exonic regions from the C1 inhibitor gene, separated by an intron. The genomic segments surrounding the duplication, i.e. exon 17 with flanking intronic sequences, were amplified from DNA from the proband as well as from control DNA, and subsequently cloned into the vector in between the two C1 inhibitor gene exons. The minigene constructs are expressed by transient transfection into cultured cells, and the splicing pattern evaluated by quantitative real-time PCR, gel separation and sequencing.¹⁴⁹

3.3.4 MTTP and PLIN2 constructs

MTTP promoter constructs

Minimal promoter constructs spanning -179 to -148, including the rs1800804T>C polymorphic site (-164T>C) in the *MTTP* promoter, were cloned into the pGL3-promoter vector, containing a generic promoter sequence to generate transcriptional activity. Longer promoter constructs covering the complete proximal promoter (-593 to +27) and both the rs1800804T>C (-164T>C) and rs1800591G>T (-493G>T) polymorphic sites, were cloned into the pGL3-basic vector. The constructs contained either the major or the minor allele(s) of rs1800804T>C and rs1800591G>T. HepG2 cells were co-transfected with either of the above vectors and the pRL-TH vector used as control, and the luciferase and Renilla luciferase activity were measured 48 h after transfection, reflecting the relative transcriptional activity mediated by each allele.

PLIN2 constructs

Construct expressing the human PLIN2 gene was made by inserting PLIN2 cDNA into vector, also containing enhanced green fluorescent protein for detection and stably transfected into HEK 293 cells. Site-directed mutagenesis was used to introduce the rs35568725 (Ser251Pro) polymorphism in the plasmid, and verified by sequencing.

3.4 IN SILICO ANALYSIS OF DNA & PROTEIN

Evolutionary Conserved Region (ECR) browser

(paper II and IV)

The ECR browser is a tool exploring the sequence conservation across species. The browser displays orthologous sequences and the degree of conservation by comparing the genome from various species including humans, rodent, fish etc.¹⁵⁰ The ECR browser was used in paper II and IV to confirm conserved regions in the *MTTP* and *PLIN2* genes.

MatInspector

(Paper I and IV)

MatInspector is a software tool for identifying putative protein and transcription factor binding sites in DNA sequences. Rather than just screening for consensus sequences, MatInspector uses weight matrices implementing the nucleotide composition and the position of the consensus alignment in the target sequence. The program also filters by similarity in the core sequence, and incorporate a conservation index for each nucleotide in order to narrow down the search result.¹⁵¹ MatInspector was used in paper I to explore putative transcription factor binding sites across the 1800804T>C polymorphic region in *MTTP*, and in paper IV to identify putative functional regions in *PLIN2*.

Regulatory Analysis of Variation in Enhancers (Raven)

(Paper IV)

Raven is a tool for identification of genetic variations in putative regulatory regions. The method uses a weight matrix in combination with phylogenetic footprinting and an estimation of the effect of the polymorphism, in order to identify functional genetic variations.¹⁵² Raven was used in paper IV to identify putative functional polymorphisms in *PLIN2*.

Clustal W

(Paper II and IV)

Clustal W¹⁵³ was used to explore degree of conservation in amino acid positions in *MTTP*, i.e. missense mutations causing ABL (paper II), and the degree of conservation at the polymorphic residue aa251 (rs35568725/Ser251Pro) in *PLIN2* (paper IV).

PHYRE and 3D JiqSaw are two different programs used for protein structure prediction. PHYRE uses multiple templates of known 3D structures to estimate the folding of the query (user submitted) sequence. The method is based on the observation that only a limited number of protein folds exist in nature, and even remotely homologous protein sequences adopt similar structures. The PHYRE program combines several different fold recognition algorithms to statistically calculate the mutational propensity for each amino acid in a given sequence in order to create a *profile*. This is done for deposited protein sequences derived from different databases, and for the query sequence. In order to construct the 3D model, PHYRE aligns the profiles (*profile-profile alignment*), rather than just comparing plain sequences. The method allows for high accuracy models even if there is a low sequence identity, but shows limitations in modelling the effect of point mutations.¹⁵⁴

3D JiqSaw uses a simpler method compared to PHYRE. The 3D Jiqsaw splits the sequence into domains and then compares each fragment against sequences in protein databases. An algorithm is used to construct the secondary structure based on the similarity between the query sequence and known secondary structures.¹⁵⁵

PHYRE was used in paper II to model the 3D structure of MTTP and to explore the relative position of four missense mutations causing ABL. The 3D JiqSaw was used in paper IV to model the impact of the missense polymorphism rs35568725 (Ser251Pro) on the secondary structure of PLIN2.

USCF Chimera¹⁵⁶ is a program used for visualisation of protein models, and was used to graphically illustrate the protein models predicted by PHYRE and 3D JiqSaw.

3.5 PROGRAMS FOR GENETIC ANALYSIS

SAS

SAS 9.1 and 9.2 software (SAS Institute Inc., Cary, NC, USA) has been used to perform most of the genetic analysis in this thesis.

PHASE

PHASE is a software for estimating haplotypes from unphased genotype data without knowing the parental genotypes. The method uses a Bayesian method for haplotype reconstruction by calculating the posterior distribution of haplotypes, conditional the assumptions of what sort of patterns of haplotypes we would expect to observe in a population with the observed genotype data. The implemented algorithm uses a Markov chain-Monte Carlo method to establish a theoretical haplotype distribution.^{157, 158}

HaploView

HaploView is a software package for calculating linkage disequilibrium and haplotype patterns from primary genotype data. The program has modules for selecting polymorphisms, i.e. tagged polymorphisms, to cover the known genetic variation in a given genomic region.¹⁵⁹

METAL

METAL is a tool for meta-analysis of two or more individual studies. The program calculates the effect size across study samples, using either the test statistics and standard errors or the p-values.¹⁶⁰

3.6 STATISTICAL METHODS

Descriptive statistics, including univariate analysis, scatter diagram, histogram and box-plots, and frequency tables were performed for the variables and parameters assessed in this thesis. When appropriate, continuous variables and parameters that did not follow a normal distribution were log-transformed using the natural logarithm or the base of 10, prior analysis. Genotype frequencies were tested for adherence to Hardy-Weinberg equilibrium using the exact test, and pairwise linkage disequilibrium was estimated using a maximum-likelihood expectation maximisation algorithm implemented in HaploView, and expressed as D' and r^2 . Differences between two groups were tested with Student's t-test (mostly unpaired). Mann-Whitney U test (Mann-Whitney-Wilcoxon/ Wilcoxon rank-sum test) was used to test independence between two groups in situations with small number of observations or continuous data not adherent to normal distribution. The differences in related samples, e.g. allele-specific gene expression, were assessed by taking the \log^{10} -transformed ratio between amount of common and minor alleles [$\log^{10} (\text{minor allele (\%)} / \text{major allele (\%)})$], and then tested for deviation from zero by one sample Wilcoxon signed-rank test. Differences in means of continuous variables between three groups (i.e. genotype groups) were tested by different general linear models, including analysis of variance (ANOVA), analysis of covariance (ANCOVA), and multivariate analysis of variance (MANOVA), linear regression and multiple linear regression. Correlation between two continuous variables were tested with Pearson's correlation. Association between outcome and independent variable(s) were tested by logistic linear regression and multiple logistic regression. Association between ordinal parameters (i.e. scores) and independent variable(s) were tested by generalised linear models. Meta analyses were performed using Metal (see paragraph 3.5).

4 RESULTS & DISCUSSION

4.1 MTTP POLYMORPHISMS

The three *MTTP* polymorphisms rs1800591G>T, rs1800804T>C and rs3816873 (Ile128Thr) are in complete allelic association ($D'=1$, $r^2=1$),^{104, 106} and numerous association studies have been performed with these polymorphisms and various trait including CVDs, plasma lipid profiles, insulin resistance and type 2 diabetes, longevity, and liver steatosis, as summarised in **tables 2-4** shown above. Evident from these tables is that there is no single, clear, and replicated trait associated with these polymorphisms. The heterogeneous results may be explained by different ethnicity, environmental factors, different aetiology, and lack of power due to small sample sizes. Nevertheless, as shown below, the minor alleles of the promoter polymorphisms result in decreased transcription of *MTTP*, and the missense polymorphism rs3816873 have been shown to reduce the thermal stability of *MTTP*.¹⁶¹

4.1.1 Allele-specific *MTTP* expression in human (Paper I and III)

Pyrosequencing technology can detect small differences in the relative gene expression from each chromosome (or allele) with high accuracy. The *MTTP* expression was measured in human samples heterozygous for the polymorphisms rs1800591G>T, rs1800804T>C and rs3816873 (Ile128Thr), allowing for detection of any imbalance in gene expression in the same individuals, minimising *cis*- and *trans*-acting effects. The allele-specific *MTTP* expression was measured in cDNA generated from left ventricular biopsies, liver and macrophages, showing a significant reduced expression of the minor allele compared to the major allele in all three tissues (mean % \pm SD of minor allele in heart: 23.4 \pm 7.3, $p=0.004$, $n=9$; liver: 46.3 \pm 2.4, $p=0.001$, $n=12$; and macrophages: 8.1 \pm 3.8, $p=0.031$, $n=6$). Results were normalised to a standard curve constructed from amplified cDNA material, homozygous for the major or minor alleles, respectively, and mixed in different ratios. DNA from each individual was used as control and generated a signal ratio of 1 (50% of each allele) reflecting the two chromosomes.

Special interest was focused on the cardiac *MTTP* expression as it has been shown that cardiac *MTTP* constitutes a mechanism by which the myocardium can get rid of surplus of lipids that otherwise may cause lipotoxicity. The influence of the promoter polymorphisms (rs1800591G>T and rs1800804T>C) on cardiac *MTTP* expression, measured using Affymetrix exon array, was investigated in 126 biopsies from human left ventricle. There was a clear association with decreased *MTTP* expression with presence of the minor alleles (mean \pm SD arbitrary units: homozygous for major alleles, 5.1 \pm 0.4, $n=73$; heterozygotes, 5.0 \pm 0.3, $n=42$; and homozygous for minor alleles, 4.7 \pm 0.3, $n=11$, $P=0.001$).

4.1.2 Cell line studies of *MTTP* polymorphisms

(Paper I)

To further investigate the impact of the promoter polymorphisms on *MTTP* expression human hepatoma cell line (Hep G2) were transiently transfected with *MTTP* promoter constructs containing the Luciferase gene for detection. Transfections with minimal promoter construct spanning only the rs1800804T>C (-164T>C) polymorphism resulted in a 30% decrease in Luciferase activity for the minor C-allele compared to the major T-allele ($P=0.004$). Constructs containing the complete proximal promoter (-593 - +27), and thus both the rs1800591G>T (-493G>T) and rs1800804T>C (-164T>C) polymorphic sites, also conferred a 30% decrease in expression for the minor alleles compared to the major alleles ($P=0.02$).

Insulin downregulates the *MTTP* expression and was used as a control of the activity of the longer promoter constructs. Transfected cells were treated with 100 nM insulin, which further decreased the promoter activity by 30% compared to untreated cells ($P=0.004$). The differences in promoter activity between constructs containing either the major alleles or the minor alleles were attenuated by insulin treatment.

The rs1800804T>C polymorphic site contains both a putative C/EBP response element, and a SRE. MatInspector detected a C/EBP response element in the sequence containing the major T-allele but not for the sequence containing the minor C-allele. MatInspector did not detect any SRE across to the rs1800804T>C polymorphic site but earlier studies have shown that a SRE, similar to the one found in the LDL-receptor promoter, is present across this polymorphic site. EMSA experiments were used to investigate the binding of C/EBP and SREBP transcription factors to the rs1800804T>C polymorphic region. The major T-allele bound more strongly to nuclear factors compared to the minor C-allele. The binding of nuclear factors to the T-allele could be competed with x100 excess of unlabelled C/EBP consensus oligonucleotide.

The SRE in the LDL receptor gene binds SREBP-1. EMSA was performed with nuclear extract from liver cells overexpressing SREBP-1 and an antibody against SREBP-1. The result showed that the LDLR-SRE consensus oligonucleotide binds nuclear proteins and a super shift was generated when SREBP-1 antibody was supplemented. However, any SREBP-1-specific binding to the oligonucleotides comprising the rs1800804T>C polymorphic region was not detected.

The effect of C/EBP on the allele-specific transcriptional activity was further investigated by co-transfection with the *MTTP* constructs (-593 to +27) and C/EBP expression vectors. HeLa cells were used as they do not have any endogenous expression of *MTTP*, minimising the background signals. Co-transfection with C/EBP α and C/EBP δ , respectively, increased the relative transcriptional activity for the construct containing the major alleles (rs1800591G/rs1800804T) compared to the minor alleles (rs1800591T/rs1800804C). C/EBP β did not significantly influence the transcriptional activity.

4.1.3 *MTTP* polymorphisms & coronary heart disease (Paper I and III)

In the case-control study INTERGENE, the minor allele of rs1800804T>C was associated with higher risk of coronary heart disease, OR: 1.76, 95%CI: 1.03-2.99. In a meta-analysis of INTERGENE and previous results from the West of Scotland Coronary Prevention Study (WOSCOPS) and Uppsala Longitudinal Study of Adult Men (ULSAM) the minor allele(s) of rs1800591G>T/rs1800804T>C was associated with increased coronary heart disease with Mantel-Haenszel OR of 1.64 (1.20-2.25) with Cochran-Mantel-Haenszel Statistic of 9.49 ($P=0.002$). The odds ratios was homogenous across all studies ($P=0.14$, Breslow-Day test). In the Framingham Offspring study and the ECTIM study there was no association between rs1800591G>T and CVDs (see **Table 2**). There are no reports of association between genetic markers mapped to *MTTP* and CVDs in GWAS.

Our functional studies clearly show that minor alleles of rs1800804T>C and rs1800591G>T result in decreased *MTTP* expression in human heart, and in a protein with reduced structural stability and weakened binding to lipoprotein particles mediated by the polymorphism rs3816873 (Ile128Thr).¹⁶¹ Decreased cardiac *MTTP* expression result in lipid accumulation and cardiac dysfunction in mice.¹⁴ Moreover, the majority of the association studies concerning plasma lipids show no/or decreased plasma lipid concentration among carriers of the minor alleles, compared to major alleles. Against this background we hypothesised that the *MTTP* polymorphisms negatively influence the cardiac function, independent of plasma lipid concentration. The association studies of CVDs include several different aetiologies, and the investigated genetic variant of *MTTP* may be a modifier of disease, related to severity of disease, ischemic damage and heart failure, which will be hard to identify in studies with a broad spectrum of CVDs. Therefore we investigated whether the *MTTP* polymorphism(s) (rs1800804T>C) and hence a decreased cardiac *MTTP* expression influence different parameters of cardiac function in the CEVENT study. The CEVENT study includes 468 patients with suspected coronary artery disease undergoing extensively characterisation of their cardiac function including echocardiography and myocardial perfusion scintigraphy. The minor allele of rs1800804T>C was associated with decreased ejection fraction, decreased tissue velocity, poorer heart muscle motion, larger infarct area and severity, and reduced reversibility and reversibility mass (see paper III).

Other studies have shown that MTTP and apoB are important for transport of excessive lipids from the heart, and that cardiac lipid accumulation may cause cardiac dysfunction,^{10, 11, 14, 15, 49, 51, 53, 162} which are in agreement with our findings. Even if our findings need confirmation in larger study samples, we suggest that a decreased cardiac *MTTP* expression in human may result in lipid accumulation and adverse cardiac manifestations. ABL-patients and patient on MTTP inhibitors should hence undergo detailed monitoring of the cardiac performance to detect any MTTP-related cardiac abnormalities.

4.2 ABETALIPOPROTEINEMIA, ABL

In paper II we characterised two patients suffering from Abetalipoproteinemia (ABL), and performed detailed genetic investigations to pin point the disease-causing mutations.

4.2.1 Two patients with loss-of-function mutation in MTTP (Paper II)

Proband 1 –MTTP missense mutation

Proband 1 and his first degree relatives were sequenced for *MTTP*. The proband was found to be homozygous for a C to T transition (NM_000253.2:c.1655C>T) causing substitution of proline to leucine at amino acid (aa) position 552 (p.Pro552Leu) in exon 13 (**Figure 4**). Both parents are heterozygous carriers of the NM_000253.2:c.1655C>T mutation, which was absent in a series of 100 consecutive control DNAs (data not shown).

The p.Pro552Leu mutation in exon 13 is located in the α -helix domain covering aa298-603, predicted to bind PDI, which is required for functional MTTP.⁵⁶ Proband 1, carrying the p.Pro552Leu mutation shows relatively mild symptoms and was diagnosed at 15-years-of-age. There has been three other ABL-transmitting missense mutations in exon 13 reported. The p.Arg540His mutation (NM_000253.2:c.1619G>A) has been shown to disrupt the interaction with PDI, and the patient displayed classical ABL symptoms and was diagnosed by the age of 17.¹⁶³ The other reported missense mutations in exon 13 include the p.His529Arg (NM_000253.2: c.1586A>G) and the p.Ser590Ile (NM_000253.2:c.1769G>T) mutations. The substitution at aa529 was detected in a 6-months-old ABL-patient with severe ABL symptoms and signs of fatty liver,¹⁶⁴ while the aa590-substitution has been reported in two unrelated ABL-patients both diagnosed in adulthood with mild symptoms.¹⁶⁵ Structural analysis of the positions of these four missense mutations shows that aa529 and aa540 are in close proximity, and separated from aa552 and aa590 by a three-turn α -helices bundle (**Figure 5**). Importantly, the p.Pro552Leu and p.Ser590Ile mutations are associated with milder phenotypes than p.Arg540His and p.His529Arg, which may suggest that these amino acid pairs are positioned at different functional domains.

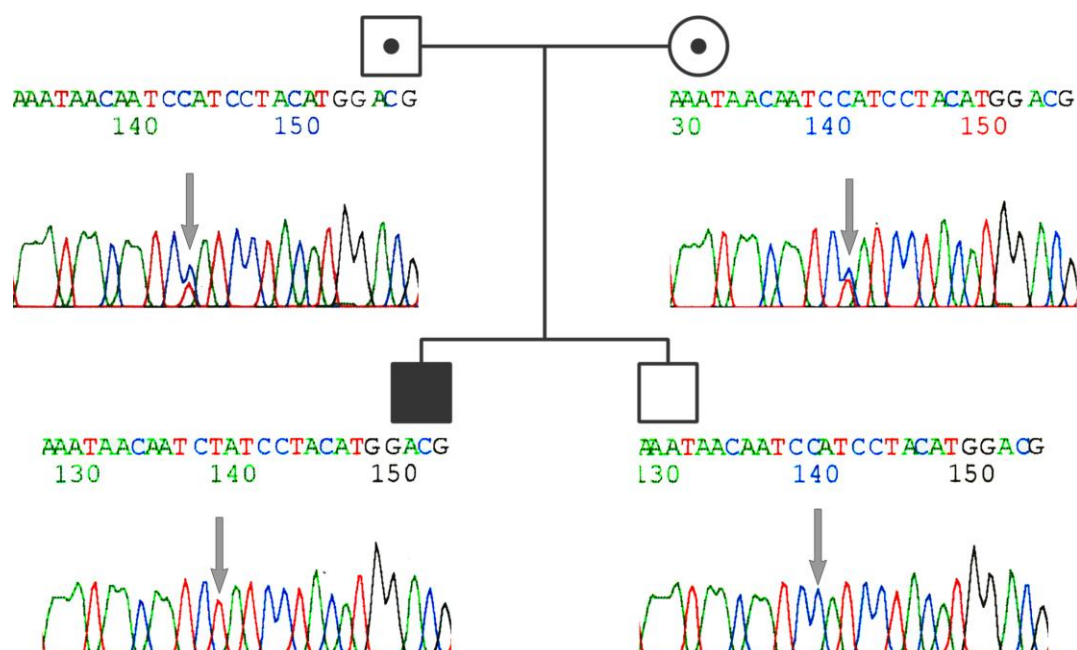


Figure 4. Family tree of proband 1 (NM_000253.2:c.1655C>T).

The family tree of proband 1 with the NM_000253.2:c.1655C>T mutation indicated with grey arrows. Sequences show bp 1644-1669 according to NM_000253.2.

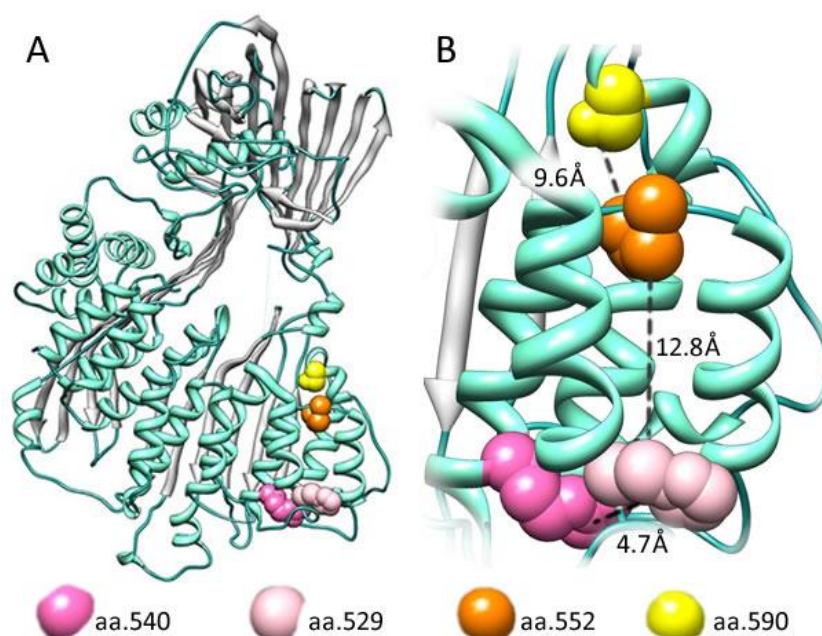


Figure 5. Structure model of MTTP.

A shows the predicted 3D structure of MTTP with highlighted positions of four reported ABL-missense mutations, including the present one (p.Pro552Leu). Yellow spheres shows position of p.Ser590Ile, orange spheres p.Pro552Leu, light pink p.Arg540His and dark pink p.His529Arg. **B** shows enlargement of **A**, where the mutations are located. Dotted lines indicate the distances between the amino acid residues measured in Ångström (Å).

Proband 2 – *MTTP* splicing mutation and uniparental disomy

An intestinal biopsy was obtained from proband 2, and subsequent analyses of the cDNA using gel electrophoresis and sequencing, revealed skipping of exon 17 (**Figure 6A and B**). Sequencing of the exons and flanking intron segments showed that the proband is a homozygous carrier of a T duplication in the splice junction of intron 17, NM_000253.2:c.2342+2dup (**Figure 6C**). The mother is heterozygous for the same duplication while the father is not a carrier (**Figure 6C**). No other mutations were found in the *MTTP* gene of these individuals by sequencing.

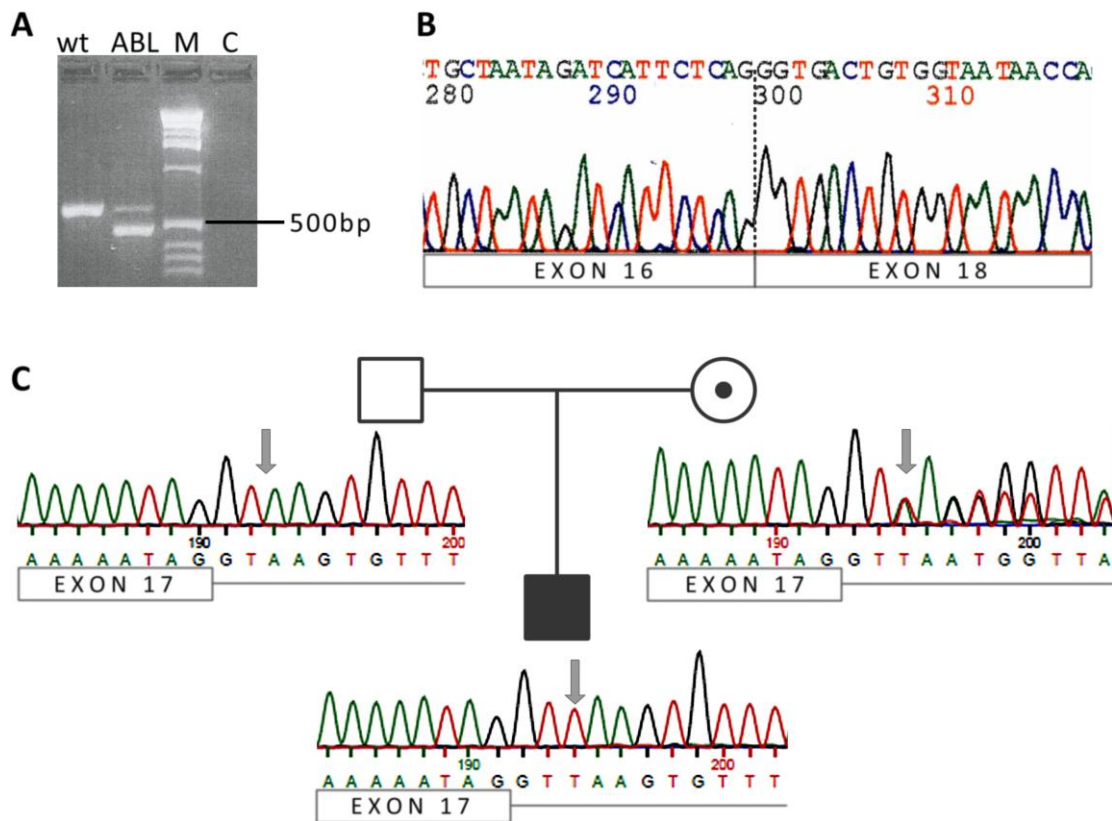


Figure 6. Genetic analyses of proband 2 with family.

A shows gel separation of amplified cDNA generated from RNA, isolated from intestinal biopsy. wt: control cDNA, ABL: cDNA from proband 2, M: marker, C: negative control. **B** shows the cDNA sequence from proband 2 showing skipping of exon 17. **C** is the family tree with sequences of the exon/intron border of exon 17 in *MTTP*. Grey arrows indicate site of insertion (NM_000253.2:c.2342+2dup).

To examine if the identified insertion causes skipping of exon 17, *in vitro* studies were performed using a splicing reporter minigene assay. The construct containing the wild type sequence, results in a longer fragment of 360 bp containing exon 17, while the construct harbouring the duplication of T, NM_000253.2:c.2342+2dup, results in a strong short fragment of 235 bp, lacking exon 17, and a weak fragment of 360 bp (**Figure 7**). The duplication of T, in the 5' splice site of intron 17 in *MTTP*, thus causes skipping of exon 17. The truncated transcript is predicted to result in a premature stop codon, p.Thr788X. However, there seems to be traces of exon inclusion (**Figure 6A and 7B**). Considering the symptom of proband 2 the remaining full-length *MTTP* seems to maintain some expression of functional *MTTP*.

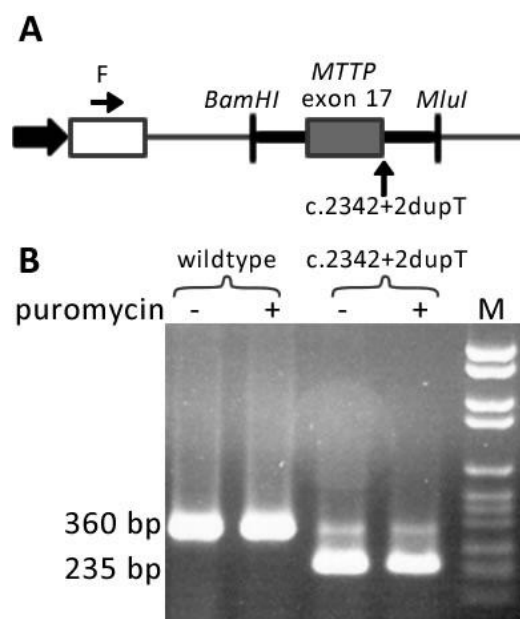


Figure 7. Splicing assay of *MTTP* mutation

A shows a schematic representation of the pCAS-2 minigene used in the functional splicing assay. Exon 17, with and without the duplication of a T in the splice junction (NM_000253.2:c.2342+2dup) of *MTTP* (dark grey box) together with the 5' and 3' intronic flanking sequences (thick lines), was cloned into the pCAS-2 minigene. The plasmid is a pcDNA3.1-based vector containing 2 exons derived from the *SERPING1/CINH* gene (white boxes), separated by their natural intron (thin line). Transcription is driven by the human cytomegalovirus immediate-early promoter/enhancer (black arrow to the left). The location of the amplification primers (F and R) are shown by arrows. **B** shows the PCR analysis of the spliced transcripts expressed in HeLa cells from the wild-type and mutant pCAS-2 minigene constructs. Sequencing of the 360 bp and 235 bp long PCR products showed that they correspond to inclusion and exclusion of exon 17, respectively.

As the proband is homozygous and the mother heterozygous for the NM_000253.2:c.2342+2dup mutation, while no aberration could be detected in the DNA from the father, paternity testing was performed. Fatherhood was verified using microsatellite markers covering chromosomes 13, 18, 21, X and Y (data not shown). To exclude deletion of the genomic region comprising the *MTTP* gene, MLPA was used. The fragment analysis could not detect any deletion across the exons in *MTTP* in the DNA from the affected proband or the parents. As no deletion was detected, haplotype analysis of chromosome 4 was performed using microsatellite markers to establish the paternal and maternal origin of the homologues. The analysis showed that the proband has inherited both chromosomes 4 from the mother, and that the 4q12-26 region is inherited from one homologue, while the telomeric regions constitute both of the mother's chromosomes 4. The proband has hence uniparental disomy (UPD) with a combination of iso- and heterodisomy. The interstitial isodisomic region stretches across the *MTTP* region located at 4q22-24, and therefore is the proband homozygous for the NM_000253.2:c.2342+2dup mutation. **Figure 8** schematically shows how UPD may have arisen in this particular case.

UPD is estimated to occur in 1 out of 3500 live births,¹⁶⁶ but UPD with normal karyotype is generally not detected unless the co-occurrence of any rare monogenic syndrome.¹⁶⁷ There are approximately 50 reports of recessive disorders as a result of UPD.¹⁶⁸ Taking into account both the infrequency of ABL and the rarity of UPD, it is interesting to note that another case of UPD associated with ABL has been reported.¹⁶⁹ This patient has maternal isodisomy of 4q21-35 but biparental inheritance of 4p16-q13, as compared to our result where the proband carries only maternal homologues. Recently, there was another case of chromosome 4 maternal UPD reported that is similar to the case presented here. The patient suffers from limb-girdle muscular dystrophy due to mutation in the β -sarcoglycan (*SGCB*) gene located at 4q12.¹⁷⁰ The 4q12 region showed isodisomy whereas other regions showed heterodisomy. This is similar to the case (proband 2) presented here with isodisomy in the 4q12-26 region.

Two other reports of segmental UPD of chromosome 4 have been published; a patient with disomy of 4p16.1-16.3 suffering from the recessive disorder Ellis-van Creveld syndrome,¹⁷¹ and a patient diagnosed with Trisomy 21 displaying isodisomy of 4p15-16 who also carries an extra derivate comprising der4:p11-q11.¹⁷² Moreover, there are two cases of confirmed complete isodisomy of chromosome 4, a patient suffering from congenital afibrinogenaemia,¹⁷³ and a woman diagnosed with major depressive disorder.¹⁷⁴ A third patient with complete chromosome 4 UPD has been presented but it is not clear if this patient displays isodisomy.¹⁷⁵ Except the two ABL-cases there are no shared symptoms described among reported UPDs of chromosome 4. Table 1 in paper II summarises the reported cases of UPD4, including one case of paternal UPD4 and four cases with complex disomy.

The majority of the reported UPDs are associated with imprinted syndromes where the disomy results in abnormal gene dosage and specific phenotypes.¹⁶⁷ To our knowledge there are only two genes reported to be imprinted on chromosome 4, *SFRP2* (4q31.3, maternally expressed)¹⁷⁶ and *NAPIL5* (4q22.1, paternally expressed).¹⁷⁷ However, there are no obvious relationships between these two genes and *MTTP*.

The mutation in proband 2 is the second among forty-five reported ABL-mutations that is caused by UPD. Therefore, in case of ambiguous homozygosity for an ABL-mutation, or any other gene mutation on chromosome 4, UPD should be considered.

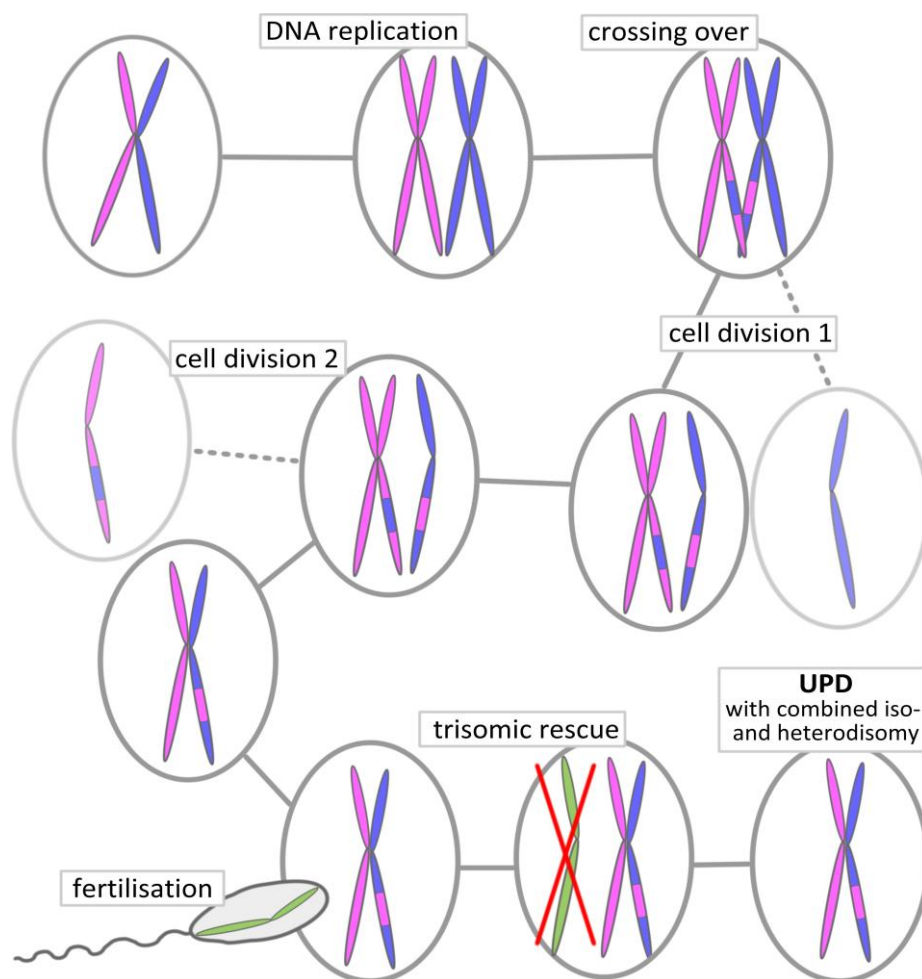


Figure 8. Uniparental disomy by trisomic rescue.

The figure shows how mitotic disjunction and trisomic rescue may result in uniparental disomy (UPD).

PLIN 2 is the most abundant lipid droplet associated protein in non-adipose tissues and is highly important for management and sequestering of intracellular lipid storages, as well as secretion of lipoproteins and hence plasma lipid concentrations. Most of the performed investigations of PLIN2 have been conducted in cell lines or in animal models, and the physiological role of PLIN2 in humans is poorly characterised. We hypothesised that genetic variation in human PLIN2 have a significant impact on the function of the protein and hence the intracellular accumulation and lipolysis of TGs, which in turn may influence the TG concentration in plasma. We therefore investigated the genetic variation in PLIN2 in relation to plasma lipid concentration.

4.3.1 Identification of a functional polymorphism in PLIN2

Eight polymorphisms across the *PLIN2* gene was genotyped in a discovery cohort (POLCA) consisting of 620 healthy middle-aged men, and tested for association with plasma lipid parameters. Three of the polymorphisms, a missense polymorphism (rs35568725/Ser251Pro), a 3'UTR polymorphism (rs1042936) and a polymorphism located 3' of *PLIN2* (rs12554335) were associated with plasma VLDL-TG and TG concentrations. The minor allele of rs35568725 (Ser251Pro) allele was significantly associated with decreased plasma VLDL-TG concentration (means, 95% CI: Ser/Ser 0.89, 0.84-0.95; Ser/Pro 0.78, 0.66-0.93; and Pro/Pro 0.33, 0.15-0.76 mmol/L, $P=0.029$). Individuals carrying the 251Pro allele also had reduced plasma TG concentration compared to non-carriers (mean 95% CI: Ser/Ser 1.35, 1.29-1.41, Ser/Pro 1.26, 1.10-1.43; and Pro/Pro 0.69, 0.38-1.26 mmol/L, $P=0.055$). In the discovery cohort the minor alleles of rs1042936 and rs12554335 were associated with increased plasma VLDL-TG ($P=0.007$ and $P=0.003$, respectively), and TG concentrations ($P=0.029$ and $P=0.006$, respectively).

The associations of these three PLIN2 polymorphisms on plasma lipids were assessed in an independent population-based cohort consisting of 1493 males and females aged 30-50 years-old from Oxfordshire, UK. The missense polymorphism Ser25Pro was significantly associated with plasma TG concentrations (means, 95% CI: Ser/Ser 1.09, 1.06-1.12; Ser/Pro 0.99, 0.92-1.07; and Pro/Pro 1.55, 0.98-2.44 mmol/L, $P=0.021$). There was no significant association between rs1042936 or rs12554335 with plasma lipids. Measurement of VLDL-TG was not available in this replication cohort.

A joint analysis combining the two cohorts was performed, and showed that the minor Pro251 allele was associated with decreased plasma concentrations ($P=0.042$). As the total number of individuals homozygous for the minor Pro251 allele is small, the effect of this polymorphism was assessed in a dominant model demonstrating that presence of the minor allele is associated with lower plasma TG concentrations ($P=0.012$).

4.3.2 Protein alignment and modelling of PLIN2 Ser251Pro

The serine residue at position aa251 in PLIN2 is highly conserved across species and located in a four α -helices bundle comprised to the PLIN2 C-terminus, which has been shown to form a stable membrane binding structure of importance for secretion of cytosolic lipid droplets.¹⁷⁸ Proline is known to disrupt α -helices as it lacks the amid hydrogen and can therefore not donate the hydrogen bond to support the α -helix, and is also a conformational rigid amino acid restricting the formation of secondary structures.¹⁷⁹ For this reason the impact of the rs35568725 (Ser251Pro) polymorphism on the secondary structure was investigated by protein modelling, showing that Pro251 does not form the hydrogen bonds to Gln248 needed to form the α -helix turn.

4.3.3 Functional studies of Ser251Pro

To investigate whether the rs35568725 (Ser251Pro) polymorphism influences the ability of PLIN2 to interact with lipid droplets peripheral monocytes were isolated from individuals homozygous for the Ser251 or the Pro251 variant. The monocytes were differentiated into macrophages and immunohistochemistry studies showed that both these PLIN2 variants bind to lipid droplets, but the Pro251-macrophages had an increased amount of intracellular lipid contents, measured as oil red O (OrO) staining per cell, compared to Ser251-macrophages (P=0.026).

Next, constructs expressing human *PLIN2* were designed, comprising either the major Ser251 variant or the minor Pro251 variant, and stably transfected into HEK 293 cells. HEK 293 cells were chosen based on their low expression of endogenous PLIN2, even after supplementation of oleic acid in the media,⁷⁷ hence minimising background signals. Two stable transfected cell lines with similar PLIN2 expression were chosen for each variant. Cells transfected with the Pro251 constructs accumulated more intracellular lipids, measured as OrO staining per cell (P=0.04) and TG (P=0.002) and had decreased lipolysis (P<0.05), compared to cells transfected with the Ser251 variant. Cells transfected with an empty vector were used as a control and had higher lipolysis compared to the cells transfected with either of the two *PLIN2* constructs (P<0.05).

5 GENERAL DISCUSSION

What and where? How and why?

It is not the amount or concentration of lipid that is the most important issue in maintaining lipid homeostasis. The questions to be asked are; *what* kind of lipids, *where* are they stored and used, as well as *how* and *why* they are stored and used.

The adipose tissue is the preferable organ for storage of excess lipids where they are stored and sequestered to be used at periods of starvations. As lipids are important energy substrate and integral part of the cell, more or less all other organs and tissues are able store lipids, even though the lipid-storage capacity is very limited and needs to be under tight control. Accumulated evidence has shown that there is a difference between lipid and lipoprotein species in their potential negative impact on cellular functions; why experimental, genetic and epidemiological studies should aim at performing detailed analysis of the lipid species and lipoprotein subclasses. Measurements of total plasma TG or cholesterol concentrations reflect TGs and cholesterol in all subclasses of lipoproteins, and are for practical reasons the most used measurements of plasma lipid concentrations. Assessment of specific subclasses of lipoproteins like VLDL1, VLDL2 and sdLDL in association studies would probably identify new loci/genes involved in lipoprotein metabolism, and decrease the need for extremely large study samples as the relative effect size of a particular lipoprotein specie is likely larger than the relative difference that can be detected in for example total TG concentration.

The fact that TG is the preferred molecule for storage of lipids is not surprising in the light of its inertness and the toxicity of its constituents, i.e. free FAs and DAG. Several studies have shown that TG accumulation *per se* is relative harmless, but chronic lipid accumulation will exceed the TG storage capacity and increase the intracellular content of toxic lipid metabolites.^{88, 180-182} Interestingly, FA species differ in their relative toxicity, which partly is explained by the level they are incorporated into TGs. Excess oleic acid and unsaturated FAs are channelled for TG synthesis and storage, whereas palmitic acid and saturated FAs result in ceramide production, production of reactive oxygen species, ER stress, and apoptosis with decreased cellular and organ function.^{88, 183-185} Similar to the dynamics between TG and its constituents, is the storage of excess cholesterol and its esterified form, CE. However, even if advanced plaques contain more free cholesterol compared to more stable plaques that predominantly contain CEs,¹⁸⁶ recent findings have shown that hydrolysis of CEs is the rate-limiting step in removal of cholesterol from foam cells (reverse cholesterol transport).¹⁸⁷ This reflects how important it is to understand the context (*what, where, how and why*) of lipid accumulation. Moreover, studies of PLIN2

in macrophages and atherosclerotic plaque have shown that PLIN2 promote lipid accumulation of TGs and CEs.^{86, 188-191} The role of PLIN2 in the formation of foam cell and progression of plaque is hence dual. PLIN2 protects the cell from toxic lipid metabolites by promoting storage of neutral lipids, but also decrease the rate of reverse cholesterol transport.^{85, 191}

When the capacity to handle incoming lipids is hampered, the cell need back-up plans to handle the lipid-induced stress. The expressions of *MTTP* and *APOB* in vital organs as the heart and kidney appears to be one mechanism by which these organs can get rid of surplus lipids, ease the stress, and restore lipid dynamics.^{13, 14}

The challenge is not to address one of the question *where*, *what*, *how*, and *why*, but to design experiment investigating several or all of them, and how they are related.

6 CONCLUSIONS

- The minor alleles of the *MTTP* promoter polymorphisms rs1800591G>T and rs1800804T>C are associated with decreased expression of *MTTP*, mediated by allele-specific binding of transcription factors to the 1800804T>C polymorphism.
- Decreased *MTTP* expression influences intracellular lipid handling, and appeared to have a detrimental effect on cardiac function, which is why patients with Abetalipoproteinaemia or patients treated with *MTTP* inhibitors should undergo detailed monitoring of their cardiac function.
- The p.Pro552Leu and NM_000253.2:c.2342+2dup mutations in *MTTP* cause Abetalipoproteinaemia. The position of p.Pro552Leu, together with the positions of three other Abetalipoproteinaemia-transmitting mutations, reflects different functional domains of *MTTP*.
- NM_000253.2:c.2342+2dup causes exon skipping of exon 17 in *MTTP*. Homozygosity for this duplication in the proband is caused by uniparental disomy, likely mediated by the mechanism trisomic rescue.
- The missense rs35568725 (Ser251Pro) polymorphism in Perilipin 2 results in increased intracellular lipid storage, reduced lipolysis, and hence lowers plasma lipid concentrations.

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