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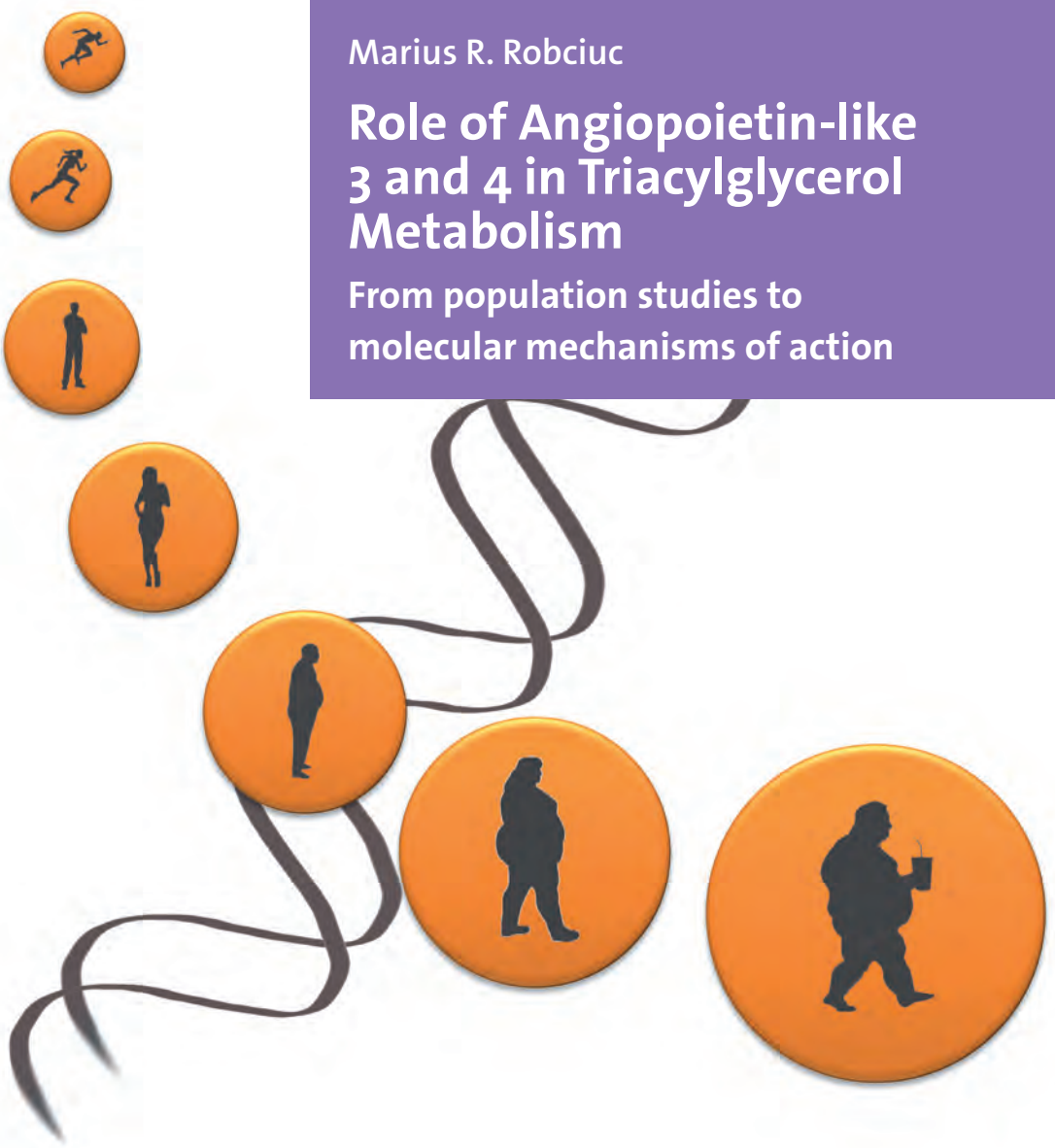
RESEARCH

Marius R. Robciuc

Role of Angiopoietin-like 3 and 4 in Triacylglycerol Metabolism

From population studies to molecular mechanisms of action



RESEARCH 83/2012

Marius R. Robciuc

ROLE OF ANGIOPOIETIN-LIKE 3 AND 4 IN TRIACYLGLYCEROL METABOLISM

From population studies to molecular mechanisms of action

ACADEMIC DISSERTATION

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Department of Chronic Disease Prevention,
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and
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Abstract

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Triacylglycerols are the most efficient molecules for storing energy in living organisms. Mammals obtain triacylglycerols either from the diet or they can synthesise them *de novo*, mainly in the liver. Gut and liver secrete triacylglycerols into the circulation packed in lipoproteins, such as chylomicrons and very low density lipoproteins, to be transported to other tissues. For storage, cell triacylglycerols are deposited in dynamic organelles called lipid droplets and in vertebrates adipocytes evolved as specialized cells to store large amounts of triacylglycerols. Excessive accumulation of triacylglycerols in circulation (hypertriglyceridemia) and/or in adipose tissue (obesity) are major risk factors for type 2 diabetes and cardiovascular diseases, two major morbidities worldwide.

The major enzymes governing the triacylglycerol metabolism are lipases, e.g. pancreatic lipase, lipoprotein lipase (LPL), hormone sensitive lipase and adipose triglyceride lipase. They catalyze the hydrolysis of triacylglycerols to release free fatty acids that are utilized further mainly for energy production or for storage.

Angiopoietin-like (Angptl) proteins are a family of secreted factors suggested to be implicated in the regulation of lipid partitioning in the body. Of these, Angptl3 and Angptl4 are potent regulators of LPL activity and plasma triacylglycerol concentration. In addition, they are suggested to enhance the breakdown of triacylglycerol stores from adipose tissue in mice.

This thesis extends our knowledge about the role of Angptl3 and Angptl4 in human triacylglycerol metabolism and provides mechanistic insights into the function of Angptl4 at cellular level.

Most of the information about the role of Angptl3 and Angptl4 in human metabolism has been obtained using genetic analysis approaches. Because these proteins are highly processed at the post-transcriptional level and are secreted the aim was to measure the levels of Angptl3 and Angptl4 in plasma/serum and identify the relevant clinical correlates. For this two quantitative ELISAs were developed and validated to measure human Angptl3 and Angptl4. Studies performed using several population samples showed that circulating levels of Angptl3 and Angptl4 have no or minor effects on plasma triacylglycerol levels. In the case of Angptl3 only complete absence of the protein in plasma, due to a nonsense mutation, generated dramatic changes not only in triacylglycerols but also in all major plasma lipoproteins and lipids, a phenotype referred to as familial combined hypolipidemia.

A role for Angptl4 in human obesity was suggested for the first time in this thesis by the study of monozygotic twins discordant for obesity. Serum Angptl4 and adipose tissue *ANGPTL4* mRNA levels are significantly decreased in obese twins as compared with their non-obese co-twins. In vitro experiments using human adipocytes revealed that Angptl4 significantly increases the breakdown of triacylglycerol stores, a mechanism that might explain the observations in human subjects.

Data obtained using animal models and human genetics clearly show that Angptl4 inhibits LPL activity and may modulate plasma triacylglycerol levels. Because the circulating levels of Angptl4 apparently do not influence LPL activity and plasma triacylglycerols, a question emerged whether Angptl4 can have more subtle roles at tissue level. In this thesis, studies using skeletal muscle cells in culture provided evidence that Angptl4 is part of a negative feedback mechanism that is likely to prevent the overload of cells with triacylglycerols. The data showed that free fatty acids, the products of LPL activity, act through peroxisome proliferator-activated receptor δ/retinoic X receptor to upregulate Angptl4 synthesis coupled to inhibition of LPL activity. Furthermore, Angptl4 appears to be regulated by insulin in a tissue specific manner suggesting a mechanism for insulin-mediated fuel partitioning in the body.

Taken together this, data establish Angptl3 and Angptl4 as important regulators of triacylglycerol metabolism in humans.

Keywords: Angiopoietin-like proteins, triacylglycerol, obesity, cardiovascular diseases, dyslipidemia, lipolysis.

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I Robciuc MR, Tahvanainen E, Jauhiainen M, Ehnholm C. Quantitation of serum angiopoietin-like proteins 3 and 4 in a Finnish population sample. J Lipid Res. 2010, 4:824-31.

II Smart-Halajko MC*, Robciuc MR*, Cooper JA, Jauhiainen M, Kumari M, Kivimaki M, Khaw KT, Boekholdt SM, Wareham NJ, Gaunt TR, Day IN, Braund PS, Nelson CP, Hall AS, Samani NJ, Humphries SE, Ehnholm C, Talmud PJ. The Relationship Between Plasma Angiopoietin-like Protein 4 Levels, Angiopoietin-like Protein 4 Genotype, and Coronary Heart Disease Risk. Arterioscler Thromb Vasc Biol. 2010 Nov; 30:2277-82. *these authors contributed equally to this work.

III Robciuc MR, Skrobuk P, Anisimov A, Eckel R, Alitalo K, Ehnholm C, Koistinen H, Jauhiainen M. Angiopoietin-like 4 mediates PPARδ effect on lipoprotein lipase-dependent fatty acid uptake but not on β-oxidation in myotubes. 2012. *Manuscript.*

IV Robciuc MR, Naukkarinen J, Ortega-Alonso A, Tyynismaa H, Raivio T, Rissanen A, Kaprio J, Ehnholm C, Jauhiainen M, Pietiläinen KH. Serum angiopoietin-like 4 protein levels and expression in adipose tissue are inversely correlated with obesity in monozygotic twins. J Lipid Res. 2011 Aug; 52:1575-82.

Abbreviations

ABHD5/CGI58α/β	hydrolase domain containing 5/comparative gene identification-58
AC	adenylyl cyclase
AGPAT	acylglycerol-phosphate acyltransferase
Angptl	angiopoietin-like
apo	apolipoprotein
ARF-1	ADP ribosylation factor 1
ATGL	adipose triacylglycerol lipase
BMI	body mass index
CCD	coiled-coil domain
CR	chylomicron remnants
CVD	cardiovascular disease
DGAT	diacylglycerol acyltransferase
EL	endothelial lipase
ELISA	enzyme linked immunosorbent assay
ER	endoplasmic reticulum
ERK2	extracellular signal regulated kinase 2
FA-CoA	fatty acyl-coenzyme A
FCHL	familial combined hyperlipidemia
FLD	fibrinogen-like domain
FPLC	fast performance liquid chromatography
GalNAc-T2	UDP-GalNAc: polypeptide N-acetylgalactosaminyltransferase 2
GPAT	glycerol-phosphate acyltransferase
GPIHBP1	glycosylphosphatidylinositol-anchored high density lipoprotein–binding protein 1
GWAS	genome wide association studies
HDL	high density lipoproteins
HL	hepatic lipase
HSL	hormone-sensitive lipase

HSPG	heparan sulfate proteoglycans
IDL	intermediate density lipoproteins
LDL	low density lipoproteins
LMF1	lipase maturation factor 1
LPL	lipoprotein lipase
LXR	liver X receptor
MGAT	acyl CoA:monoacylglycerol acyltransferase
MGLL	monoacylglycerol lipase
MTP	microsomal triglyceride transfer protein
NLSE	neutral lipid-synthesizing enzymes
PACE4	paired amino acid converting enzyme-4
PC	proprotein convertases
PDE-3B	phosphodiesterase 3B
PHP	post heparin plasma
PI3-K	phosphatidylinositol-3 phosphate kinase
PK	protein kinase
PLIN	perilipin
PPAR	peroxisome proliferator-activated receptor delta
RXR	retinoic X receptor
SGBS	Simpson-Golabi-Behmel syndrome
SNPs	single nucleotide polymorphisms
TRLs	triacylglycerol-rich lipoproteins
VAMP7	vesicle associated membrane protein 7
VLDL	very low density lipoproteins

1 INTRODUCTION

Triacylglycerol metabolism is currently under extensive investigation because of its critical role in the development of metabolic syndrome, obesity, diabetes and cardiovascular disease (CVD), the leading causes of death worldwide. Triacylglycerol is a safe and efficient molecule to store fatty acids, which together with glucose are the major energy source for living organism. Because triacylglycerols are highly hydrophobic, they are stored in specialized organelles called lipid droplets that exist in all eukaryotic cells. In complex organisms triacylglycerols are transported between different tissues packed in lipoproteins. In mammals, plasma triacylglycerols are present mainly in the core of the triacylglycerol-rich lipoproteins (TRLs) such as chylomicrons and very low density lipoproteins (VLDL). Mobilisation of free fatty acids from triacylglycerol stored in lipid droplets or packed in lipoproteins is achieved by specialized enzymes called lipases.

Lipoprotein lipase (LPL), bound to capillary endothelium, hydrolyzes the triacylglycerols in chylomicrons or VLDL generating remnant particles. Remnants are rapidly cleared from circulation by the liver but some of them can penetrate the vessel wall and contribute to the development of atherosclerosis (Proctor et al, 2004). Whether increased concentration of plasma triacylglycerol is an independent risk factor for cardiovascular disease has been a matter of debate for three decades. This is because hypertriglyceridemia is often part of the dyslipidemic profile including low high density lipoproteins (HDL) levels and elevated levels of small dense low density lipoproteins (LDL). It is now clear that elevated levels of triacylglycerol in plasma is an independent risk factor for the development of CVD and recently, statements from the European Atherosclerosis Society Consensus Panel and the American Heart Association have been published to update clinicians on this matter (Chapman et al, 2011; Miller et al, 2011). Severe elevations of plasma triacylglycerols are observed in patients with familial combined hyperlipidemia (FCHL) and familial hypertriglyceridemia. FCHL is the most common genetic lipid disorder associated with increased risk for CVD affecting 0.5% - 2.0% of individuals in the general population (Gaddi et al, 2007).

Chronic positive energy balance due to overnutrition and inactivity is common in our modern society and increasing. This leads to increased deposition of triacylglycerol in adipose tissue and to the development of obesity. Obesity is a serious medical condition because it increases the risk of cardiovascular disease, type 2 diabetes, and some cancers, among other health problems. When the storage capacity of adipose tissue is exceed other tissues, such as liver, skeletal muscle, heart and pancreas, become overloaded with triacylglycerols. If the oxidative and storage capabilities of the cells became saturated there is an accumulation of fatty acid intermediates, such as diacylglycerol and ceramide, that are likely to contribute to insulin resistance

(Samuel et al, 2010). Impaired insulin function leads to uncontrolled release of free fatty acids from adipose tissue, oversecretion of triacylglycerol-rich VLDL particles ($VLDL_1$) and delayed hepatic remnant clearance by the liver and impaired glucose uptake in skeletal muscle (Samuel et al, 2010).

Important discoveries in the past decade have identified many key regulators of the triacylglycerol metabolism. Understanding the detailed mechanisms by which these molecules function will facilitate the development of new therapeutic strategies to restore the imbalance of triacylglycerol metabolism in obesity, diabetes and cardiovascular disease.

2 REVIEW OF THE LITERATURE

2.1 The triacylglycerol metabolism

2.1.1 The triacylglycerol molecule

Each triacylglycerol (also called triglyceride or fat) molecule is composed of three fatty acids bound through an oxyester bond to a single glycerol molecule (Figure 1). In nature the triacylglycerols are usually composed from two or more different fatty acids that can have differences in length and degree of saturation. Naturally occurring triacylglycerols contain almost invariably long chain fatty acids with the aliphatic tail longer than 12 carbon atoms and in animals they reflect to some extent the composition of the diet (Perona et al, 2000).

Because all three polar hydroxyl groups of glycerol form ester bonds with the polar carboxyl group of fatty acids, the triacylglycerol molecule is non-polar and highly hydrophobic. The hydrophobicity of triacylglycerol molecule confers an advantage as a storage molecule when compared with glycogen and starch (the storage forms of glucose) that require hydration and thus weight more. In addition, the oxidation of triacylglycerol generates about six times more ATP as does the same quantity of glycogen. Therefore, humans that are overweight can use their triacylglycerol stores to meet their energy needs for months, whereas the glycogen stores can provide energy for less than a day (Nelson & Cox, 2005).

Although triacylglycerols serve mainly as energy reserve they also have other functions. The adipose tissue provides insulation for organisms and in some marine animals help to maintain buoyancy (Nelson & Cox, 2005). Normal triacylglycerol biosynthesis is critical for normal skin development and permeability barrier function (Stone et al, 2004). Moreover, triacylglycerol is the major fuel for brown adipose tissue to maintain body temperature during cold exposure (Bartelt et al, 2011). Because triacylglycerol is chemically relatively inert, it can protect the cells from the potentially toxic effects of excess fatty acids. This is evident in tissues with limited capacity to store triacylglycerols, such as muscle and liver, where lipotoxicity is observed as they become overloaded with fatty acids (Muoio & Newgard, 2008).

Triacylglycerols from seeds and fruits serve as valuable resources for industry, most recently as their potential to be used as biofuels.

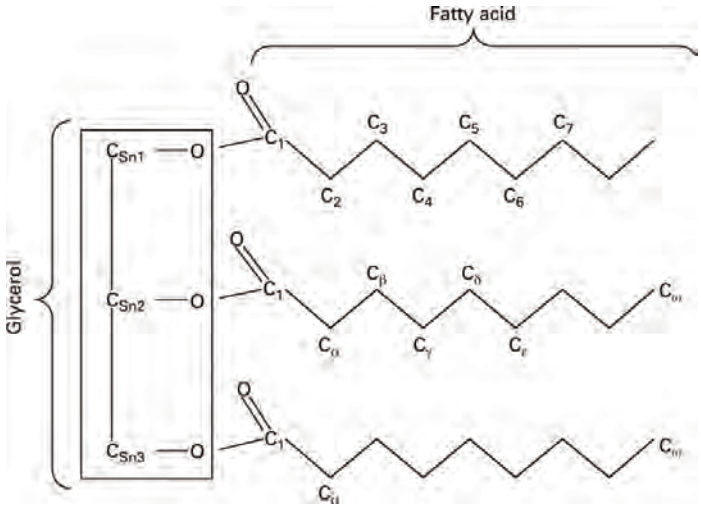

Figure 1. **Triacylglycerol molecule showing the Sn positions and the numerical and alphabetical nomenclatures of fatty acids (Manson & Weaver, 1997)** (reprinted with permision from BMJ Publishing Group Ltd)

2.1.2 Major sources for triacylglycerols

Fatty acids stored in triacylglycerols can be synthesised de novo, mainly in the liver (endogenous source), or obtained from the diet by absorption in the intestine (exogenous source).

For 2500 calorie intake daily it is generally recommended to consume 80-90 grams of fat (triacylglycerols), of which more than 95% is absorbed in the intestine, mostly in the duodenum and proximal jejunum. Before absorption by enterocytes, triacylglycerols are emulsified in bile acid micelles and hydrolysed by pancreatic lipase/colipase complex at the positions *sn*-1 and *sn*-3 to produce 2-monoacylglycerol and fatty acids (Mattson & Beck, 1956). Enterocytes can absorb 2-monoacylglycerol and fatty acids by diffusion or specific transporters not clearly defined hitherto (Iqbal & Hussain, 2009). CD36 is the best characterized lipid transporter that appears to have a key role in efficient utilisation of dietary fatty acids (Drover et al, 2005).

Fatty acid synthase is a cytoplasmic enzyme responsible for the biosynthesis of fatty acids. Mammalian fatty acid synthase is active only as an intertwined dimer with two lateral semicircular reaction chambers for fatty acid elongation (Maier et al, 2006). Each monomer contains seven protein domains required for fatty acid synthesis: acyl carrier, acyl transferase, β-ketoacyl synthase, β-ketoacyl reductase, β-hydroxylacyl dehydratase, enoyl reductase, and thioesterase. Fatty acid synthase uses the glucose intermediate metabolite, acetyl coenzyme A (CoA), to synthesize

unsaturated fatty acids such as palmitate (C16:0) and to a lesser extent longer or shorter fatty acids.

Fatty acids, from *de novo* synthesis or diet, can enter the pathway of triacylglycerol biosynthesis in the form of CoA esters and as partial acylglycerols.

The glycerol for the initial esterification may be derived in the form of 2-monoacylglycerols from lipolysis of triacylglycerols or in the form of dihydroxyacetone and glycerol-3-phosphate from glycolysis. Two major pathways for triacylglycerol biosynthesis have been described more than 50 years ago: the glycerol phosphate pathway and the 2-monoacylglycerol pathway (Figure 2). The glycerol phosphate pathway is present in most tissues whereas the 2-monoacylglycerol pathway occurs mainly in enterocytes, liver and adipose tissue. Both pathways are governed by acyltransferase and lipin enzymes that work in sequential steps shown figure 2. Acyltransferases transfer one activated fatty acid (fatty acyl-coenzyme A, FA-CoA) to the glycerol backbone whereas lipin proteins have phosphatidate phosphatase activity and catalyze the formation of diacylglycerol in the glycerol-3-phosphate pathway.

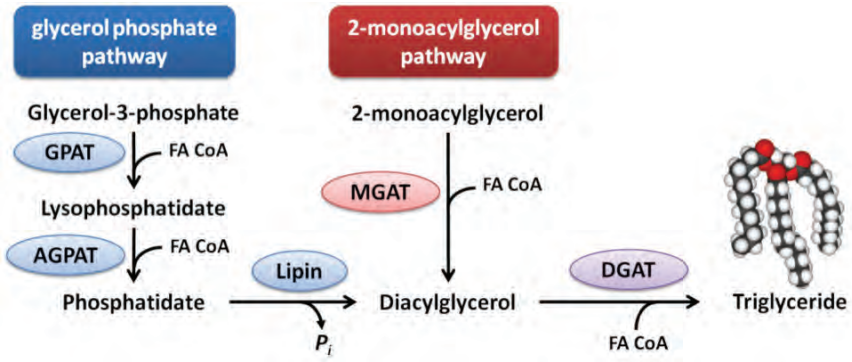

Figure 2. **Major pathways for triacylglycerol biosynthesis.** GPAT, glycerol-phosphate acyltransferase; AGPAT, acylglycerol-phosphate acyltransferase; MGAT, acyl CoA:monoacylglycerol acyltransferase; DGAT, diacylglycerol acyltransferase.

Newly synthesized triacylglycerols are thought to be released into the associated lipid bilayer and either stored in lipid droplets (in all tissues) or packed in lipoproteins (in hepatocytes and enterocytes) and secreted for transport to other tissues (Figure 3).

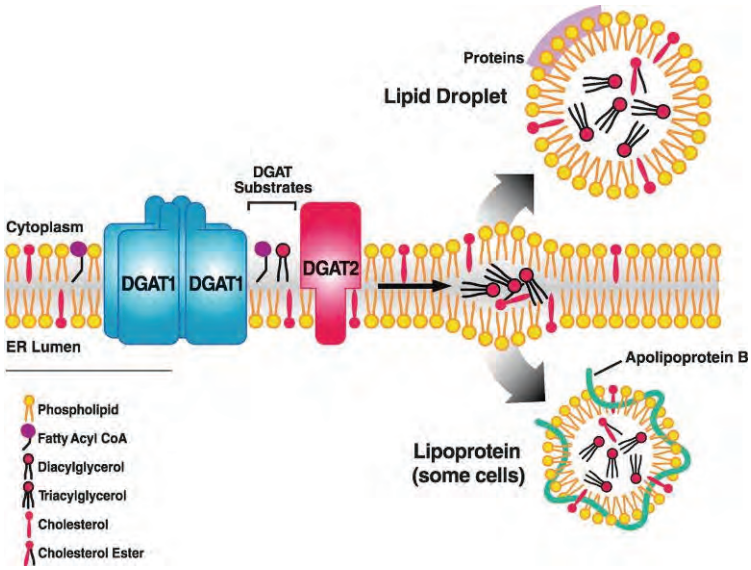

Figure 3. **Hypothetical model illustrating the role of diacylglycerol acyltransferase (DGAT) enzymes in triacylglycerol synthesis in the ER (Yen et al, 2008).** Triacylglycerol products of the DGAT reaction may be channeled into the cores of cytosolic lipid droplets or triacylglycerol-rich lipoproteins for secretion in cells such as enterocytes and hepatocytes. (Reprinted with permision from American Society for Biochemistry and Molecular Biology)

2.1.3 Storage of triacylglycerols

Lipid droplets are the major cellular organelles for the storage of triacylglycerols and other neutral lipids. Triacylglycerols are located in the core of the organelle and are separated from the aqueous phase of the cell by a monolayer of phospholipids. This structure is stabilized by structural proteins such as perilipin (Greenberg et al, 1991). In addition to structural proteins, lipid droplets are decorated with lipid-synthesis enzymes (e.g. diacylglycerol acyltransferase, DGAT2), lipases (e.g. hormone sensitive lipase, HSL), and membrane-trafficking proteins (e.g. ras-related small GTP binding protein, Rab5). Several models for the biogenesis of lipid droplets have been proposed and are depicted in figure 4. The common feature is that lipid droplets are generated at the endoplasmic reticulum (ER) membrane where neutral lipid-synthesizing enzymes (such as DGAT2) are located. As very dynamic organelles, lipid droplets enlarge by fusion and in adipocytes can reach more that 100 µm in diameter (Bostrom et al, 2007; Walther & Farese, 2009). Moreover, they

undergo fission, a process that might facilitate efficient intracellular lipolysis (Marcinkiewicz et al, 2006).

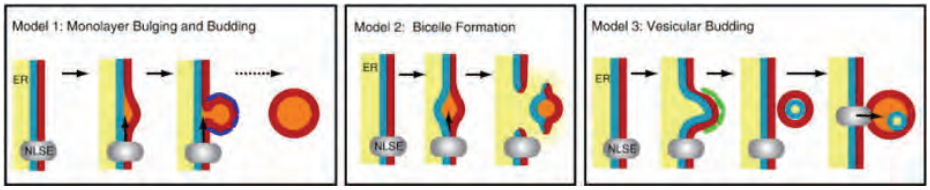

Figure 4. **Models of lipid-droplet biogenesis (Walther & Farese, 2009).** (Left) Model 1: Lipid droplet biogenesis by ER budding. Neutral lipids (orange) are synthesized by neutral lipid-synthesizing enzymes (NLSE) and bulge from the outer leaflet of the ER membrane (red). The nascent droplet may be coated by proteins (dark blue) that facilitate the budding process. (Middle) Model 2: Bilayer excision. Newly synthesized neutral lipids accumulate between the inner (blue) and outer (red) leaflets of the ER membrane and cause bulging. This entire lipid lens is then excised from the ER, leaving a transient hole in the membrane. ER contents (yellow) might leak through this hole into the cytosol. (Right) Model 3: Vesicular budding. A vesicle containing both leaflets of the ER membrane (red and blue) and a lumen (yellow) is formed by the vesicular budding machinery (green) at the ER membrane. The vesicle is subsequently tethered to the ER, where NLSEs (grey) fill the intramembranous space with neutral lipids (orange). The luminal space (yellow) is compressed, and its contents may leak into the cytosol. This process may trap luminal proteins within a compartment of the lipid droplet. (Reprinted with permission from Elsevier)

2.1.4 Intracellular lipolysis

Hydrolysis of stored triacylglycerols to glycerol and fatty acids is achieved by lipases in a process referred as intracellular lipolysis. Three lipases act at the lipid droplet surface in sequential actions for complete hydrolysis of triacylglycerol stores (Figure 5). Adipose triacylglycerol lipase (ATGL) is the rate limiting enzyme for the first step of lipolysis hydrolyzing triacylglycerol to diacylglycerol and fatty acid (Jenkins et al, 2004; Villena et al, 2004; Zimmermann et al, 2004). Hormone-sensitive lipase (HSL or LIPE) is the rate limiting enzyme for the second step of lipolysis hydrolyzing diacylglycerol to monoacylglycerol and fatty acid (Haemmerle et al, 2002; Vaughan et al, 1964). The lipolysis is finalized after the hydrolyzis of monoacylglycerol to fatty acid and glycerol by the monoacylglycerol lipase (MGLL) (Karlsson et al, 1997; Tornqvist et al, 1972; Vaughan et al, 1964). ATGL, discovered 40 years after the other two lipases, is the principal lipase responsible for basal levels of lipolysis since unphosphorylated HSL is predominately located in the cyto-

plasm (Egan et al, 1992; Lass et al, 2006). In adipose tissue, perilipin 1 is central in maintaining the basal levels of lipolysis by blocking the access of HSL to the lipid droplet surface and by sequestering the ATGL activator α/β hydrolase domain containing 5/comparative gene identification-58 (ABHD5/CGI58) (Granneman et al, 2007; Subramanian et al, 2004). Non-adipose tissues express little or no perilipin-1 and other perilipins, such as perilipin 5 in oxidative tissues, might play an important role in the translocation of the lipases to lipid droplets and their interaction with activators (Granneman et al, 2009; Lass et al, 2011).

Lipolysis is a tightly controlled process extensively investigated in adipose tissue. Catecholamines, natriuretic peptides, and insulin are considered to represent the major regulators of lipolysis in human adipose tissue (Lafontan & Langin, 2009). Adrenaline and noradrenaline bind the β-adrenergic receptors coupled to stimulatory Gs proteins and activate adenylyl cyclase leading to a rise in intracellular cyclic adenosine monophosphate (cAMP) levels. In a similar fashion natriuretic peptide binds its receptor and increases intracellular cyclic guanosine monophosphate (cGMP) levels (Sengenes et al, 2000). The increased levels of intracellular cAMP and cGMP lead to the activation of protein kinase A (PKA) and protein kinase G (PKG), respectively. PKA and PKG phosphorylate HSL and perilipin 1 (PLINA) setting the stage for HSL to bind to the lipid droplet surface and increase its activity by up to 100-fold (Krintel et al, 2008; Schweiger et al, 2006; Stralfors & Belfrage, 1985). Phosphorylation of perilipin 1 also causes the dislocation of the CGI-58 available now to fully activate ATGL activity (Granneman et al, 2007; Subramanian et al, 2004). Human ATGL is also phosphorylated in a PKA independent manner, however, the relevance of phosphorylation in the regulation of enzyme activity is unclear (Lass et al, 2011; Zimmermann et al, 2004). In contrast to catecholamines, insulin promotes cAMP degradation upon binding to the insulin receptors and stimulation of the phosphodiesterase-3B (PDE-3B) hence intracellular lipolysis is inhibited. To date, no evidence exists that cellular MGLL mRNA concentrations or enzyme activities are regulated by either hormones or the energy state of the cell (Lass et al, 2011).

Since all cells can store triacylglycerols in lipid droplets they are also equipped with the basic lipolytic machinery although some of the components and regulatory pathways are different (Lass et al, 2011).

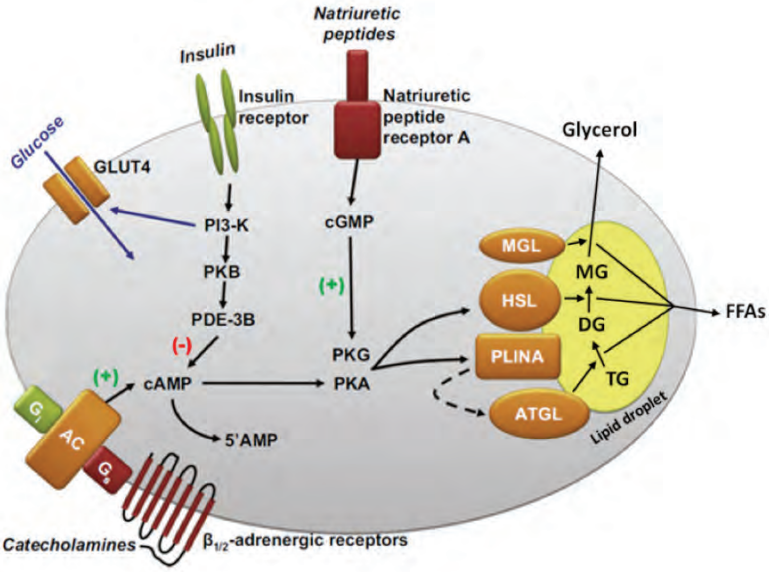

Figure 5. **Control of Human Adipocyte Lipolysis.** Binding of catecholamines to Gs protein-coupled b1/2-adrenoceptors stimulates cAMP production by adenylyl cyclase (AC) and activates protein kinase A (PKA). Insulin favors cAMP degradation through activation of phosphatidylinositol-3 phosphate kinase (PI3-K) and protein kinase B (PKB) and stimulation of phosphodiesterase 3B (PDE-3B) activity. Natriuretic peptides promote cGMP accumulation and protein kinase G (PKG) activation. PKA and PKG phosphorylate hormone-sensitive lipase (HSL) and perilipin 1 (PLINA). Adipose triglyceride lipase (ATGL) and monoglyceride lipase (MGL) are also participating in the hydrolysis of triglycerides (modified after (Langin, 2010).

2.1.5 Transport of triacylglycerols

Triacylglycerols are transported in large macromolecular complexes of lipids and proteins called lipoproteins. The function of lipoproteins is to transport water insoluble lipids such as triacylglycerols and cholesteryl esters in circulation. The structure of lipoproteins resembles that of lipid droplets with a monolayer of phospholipids stabilised by apolipoproteins such as apolipoprotein B (apoB) and a core containing neutral lipids. Triacylglycerols are mainly transported in triacylglycerol-rich lipoproteins (TRLs), namely very low density lipoproteins (VLDL) and chylomicrons. VLDL are secreted by the liver in plasma whereas chylomicrons are secreted by the small intestine into the lymph.

Chylomicrons, assembled in enterocytes, are stabilised by a shorter form of apolipoprotein B, apoB48, produced via a specific RNA editing mechanism (Powell et al, 1987). The lipidation of apoB48 has many similarities with two-steps VLDL production in the liver (Cartwright & Higgins, 2001). In the first step newly synthesized apoB48 is lipidated by microsomal triglyceride transfer protein (MTP) at the inner leaflet of the endoplasmic reticulum (ER). The second step is initiated after poorly lipidated apoB48 detach from the ER membrane to form a "primordial particle" (Mansbach & Gorelick, 2007). Further enlargement of the particle occurs by MTP-dependent lipidation or fusion with preformed lipid droplets in a process termed "core expansion" (Hussain, 2000). These steps generate pre-chylomicrons which are packed in special vesicles called prechylomicron transport vesicles (PCTV) and transported to Golgi apparatus before secretion in the lymph (Cartwright & Higgins, 2001; Cartwright et al, 2000; Siddiqi et al, 2003). The formation of PCTV is coat protein complex II (COPII)-independent and distinct from vesicles that transport proteins (Siddiqi et al, 2003). An important feature of PCTV that further distinguishes them from protein vesicles is the presence of v-SNARE and vesicle associated membrane protein 7 (VAMP7) which facilitates the fusion with *cis*-Golgi (Mansbach & Gorelick, 2007; Siddiqi et al, 2006).

VLDL is assembled within the secretory pathway of hepatocytes in two major steps (Olofsson et al, 2000) (Figure 6). The first step occurs in the rough ER where MTP lipidates apoB co- and post-translationally forming pre-VLDL (Boren et al, 1992; Sharp et al, 1993; Wetterau et al, 1992). If the lipidation is inadequate apoB is exposed to the cytosol, recognised by chaperones such as heat shock protein 70 (Hsp70) as a misfolded protein, ubiquitinated and degraded mainly via the ubiquitin–proteasome system (Fisher et al, 1997). Properly lipidated pre-VLDL is converted to $VLDL_2$ by further lipidation in the second step of VLDL biogenesis (Olofsson et al, 2000). This occurs in the smooth ER compartment during the assembly of $VLDL_2$ in Sar1/COPII vesicles. $VLDL_2$ containing vesicles are transported to Golgi apparatus, a process dependent on ADP ribosylation factor 1 (ARF-1). In Golgi apparatus $VLDL_2$ is either secreted in plasma or acquire a major proportion of triglycerides and form $VLDL_1$. Formation of $VLDL_1$ is dependent on phospholipase D1 (PLD1) and extracellular signal regulated kinase 2 (ERK2) (Andersson et al, 2006; Verges, 2010). $VLDL_1$ is also secreted in plasma and high levels are associated with insulin resistance and type 2 diabetes (Taskinen, 2003).

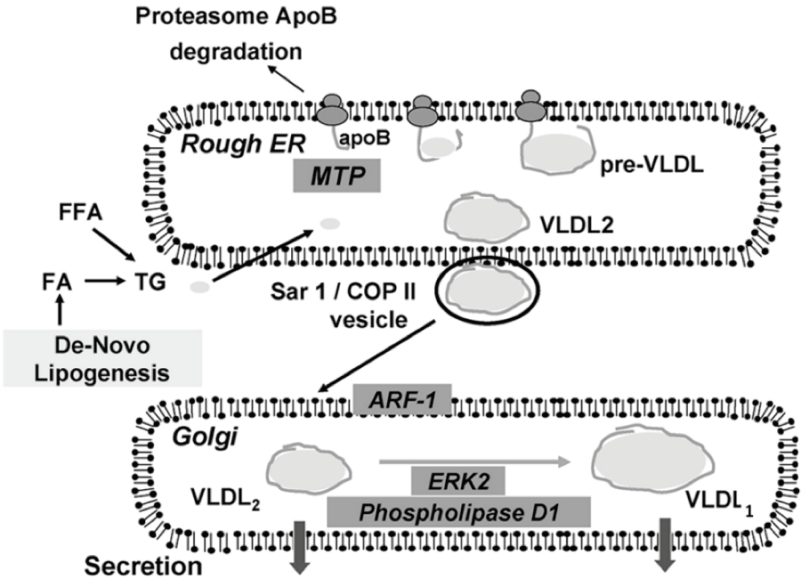

Figure 6. **VLDL assembly and secretion (Verges, 2010).** First step: in the rough endoplasmic reticulum (ER), apoB is lipidated by the microsomal transfer protein (MTP), leading to the formation of pre-VLDL. Second step: pre-VLDL is further lipidated to form VLDL2. VLDL2 exits the ER compartment by Sar1/COPII vesicles, which are directed to the Golgi apparatus. ADP ribosylation factor 1 (ARF-1) is involved in VLDL2 trafficking between the ER and the Golgi apparatus. In the Golgi apparatus, VLDL2 is converted to larger VLDL1 by addition of lipids. This step is promoted by Phospholipase D1 and ERK2. FA: fatty acid, FFA: free fatty acid, apoB: apolipoprotein B, VLDL: very low density lipoprotein, TG: triglycerides, ER: endoplasmic reticulum, MTP: microsomal transfer protein, ARF-1: ADP ribosylation factor 1, ERK2: extracellular signal-regulated kinase 2. (Reprinted with permission from Elsevier).

VLDL and chylomicrons acquire other proteins (mostly exchangeable apolipoproteins) on their surface, either intracellularly or in systemic circulation, that are critical for their metabolism. Remodelling of TRLs in systemic circulation is very complex and not completely understood but the rate limiting enzyme responsible for the delivery of triacylglycerol fatty acids to peripheral tissues is lipoprotein lipase (LPL).

2.1.6 Lipoprotein lipase

LPL is a member of extracellular lipase family that also includes pancreatic lipase, hepatic lipase and endothelial lipase (Olivecrona & Olivecrona, 2009). The active form of LPL is a head-to-tail dimer stabilized by noncovalent interactions (van Tilbeurgh et al, 1994; Wong et al, 1997). Formation of active LPL dimer is facilitated by a specific chaperone called lipase maturation factor 1 (LMF1) (Peterfy et al, 2007). LPL dimer is unstable under physiological conditions suggesting that it is spring-loaded and endowed with a built-in mechanism of self-destruction (Osborne et al, 1985). This is an important property for an enzyme that acts extracellularly but whose activity needs to be rapidly regulated (Olivecrona & Olivecrona, 2009). This property is used by the LPL inhibitor angiopoietin-like 4 (Angptl4) that interacts with the dimer and triggers the formation of inactive monomers (Sukonina et al, 2006).

LPL exerts its function bound to the luminal surface of capillaries, within heart, white and brown adipose tissue, and skeletal muscle, where it binds TRLs and hydrolyses their triacylglycerol core to release fatty acids for use by parenchymal cells (Figure 7). Because LPL is synthesised in parenchymal cells it has to be transported to the luminal side of the endothelium to function efficiently. Due to a large number of positively charged amino acid residues in its structure, secreted LPL is retained in the glycocalyx bound to heparan sulfate proteoglycans (HSPG). Glycosylphosphatidylinositol-anchored high density lipoprotein–binding protein 1 (GPIHBP1), a GPI-anchored protein produced by capillary endothelial cells, binds LPL with higher affinity than HSPG in the subendothelial space and transports it to the capillary lumen (Figure 7) (Beigneux et al, 2007; Davies et al, 2010). The mechanisms of lipolysis at the endothelium surface is not yet defined but it might involve the clustering of LPL by GPIHBP1 in caveolae (Young et al, 2011).

LPL delivers the triacylglycerols to the tissues mainly after hydrolysis of core triglycerides in TRLs. In this process, chylomicrons and VLDL are converted to chylomicron remnants (CR) and intermediate density lipoproteins (IDL) whereas triacylglycerol is hydrolysed to fatty acids and a 2-monoglyceride (Hassing et al, 2011; Morley & Kuksis, 1972). CR and IDL are rapidly cleared by the liver or, in the case of IDL, it is also converted further to low density lipoproteins (LDL). Products of triacylglycerol hydrolysis are rapidly taken up by the adjacent parenchymal tissue, although a significant amount of fatty acids is released in plasma as albumin bound fatty acids (Bickerton et al, 2007; Hultin et al, 1996). Besides its hydrolytic activity, LPL can facilitate the uptake of triacylglycerol in cells also by enhancing the endocytosis of lipoprotein particles (Fernandez-Borja et al, 1996; Merkel et al, 2002; Olivecrona & Olivecrona, 2009).

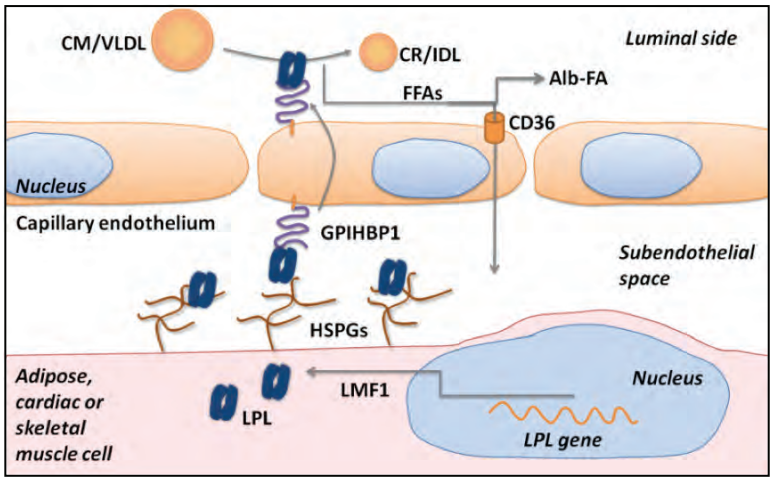

Figure 7. **LPL mediated hydrolysis of plasma triacylglycerol.** LPL is synthesized in parenchymal cells (such as adipocytes, cardiomyocytes and myofibrils), maturated by lipase maturation factor 1 (LMF1) and secreted in the subendothelial space where it binds to heparane sulfate proteoglycans (HSPGs). Glycosylphosphatidylinositol-anchored high density lipoprotein–binding protein 1 (GPIHBP1) binds LPL with higher affinity than HSPGs and transport it at the luminal site of the capillary. LPL, located probably in lipid rafts bound to GPIHBP1, engages VLDL and chylomicrons (CM) and hydrolyses their triacylglycerol to generate remnants (CR) and IDL, respectively. Most of the fatty acids generated by LPL are rapidly taken up by adjacent parenchymal tissue although a significant amount of is released in plasma as albumin bound fatty acids. The fatty acids will be locally internalized by fatty acid transporters such as CD-36.

LPL activity is pivotal in maintaining the physiological distribution of triacylglycerols among tissues in response to energy requirements and hormonal changes. For this, LPL is regulated at transcriptional, posttranscriptional, translational, and post-translational levels in a tissue-specific manner (Wang & Eckel, 2009). Studies in mice provided evidence that altered LPL expression and regulation has major metabolic consequences on macronutrient fuel partitioning, energy homeostasis, insulin action, and lipoprotein metabolism (Wang & Eckel, 2009).

2.2 Angiopoietin-like family

The angiopoietin-like (Angptl) protein family comprises of seven secreted molecules which have structural similarities with angiopoietins (Conklin et al, 1999; Kersten et al, 2000; Kim et al, 2000; Kim et al, 1999a; Kim et al, 1999b; Oike et al, 2003; Peek et al, 2002; Yoon et al, 2000). The structure of Angptls is predicted to contain an N-terminal coiled-coil domain (CCD) and a C-terminal fibrinogen-like domain (FLD) (Figure 8) (Davis et al, 2003; Mattijssen & Kersten, 2011). Biochemical analyses suggest that at least some Angptls also form oligomers via the CCD as it is the case with angiopoietins (Figure 8) (Davis et al, 2003; Ge et al, 2005; Ge et al, 2004a).

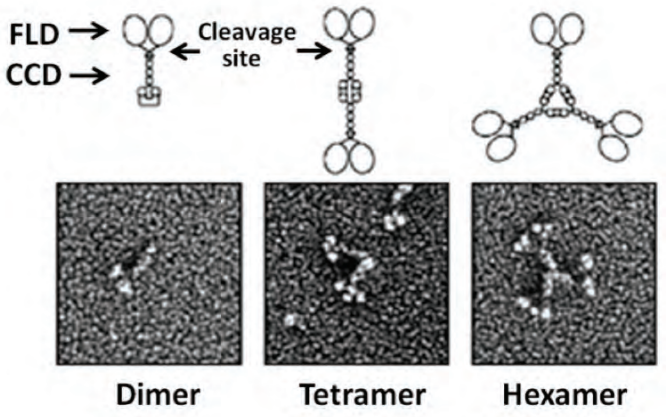

Figure 8. **Structure of angiopoietin oligomers (Davis et al, 2003).** Schematic representations of angiopoietin structures (top) and transmission electron microscopy visualization (bottom) of Angiopoietin1 oligomers. *Fibrinoge*n-like domain (FLD), Coiled-coil domain (CCD). Modified after Davis et al, 2003. (Reprinted with permission from Nature Publishing Group).

Although Angptl molecules share similar domain structures with angiopoietins, they do not bind to the tyrosine-protein kinase receptors, TIE2 or TIE1 (Augustin et al, 2009; Kim et al, 1999a; Kim et al, 1999b). FLDs of Angptls are expected to function as signaling molecules and hitherto several studies have shown that they bind and signal via integrins (Camenisch et al, 2002; Tabata et al, 2009; Zhang et al, 2006; Zhu et al, 2011). In contrast to angiopoietins, Angptls are proteolytically cleaved by proprotein convertases to release the FLD and CCD (Figure 8) (Ge et al, 2004a; Ono

et al, 2003). It was suggested that the proteolytic cleavage of Angptls can potentiate some of their functions (Chomel et al, 2009; Ono et al, 2003; Schjoldager et al, 2010), thus it can be an important regulatory step in the mechanism of action of Angptls that remains to be established. Most of the knowledge we have about Angptls comes from studies on Angptl3 and Angptl4, but it is expected that post-translational modifications like oligomerisation and cleavage play an important role for the function of other Angptls as well.

Angptls are pleiotropic molecules involved in angiogenesis, tumor metastasis, wound healing, appetite control and lipid and glucose metabolism (Goh et al, 2010; Hato et al, 2008; Kim et al, 2010; Mattijssen & Kersten, 2011). Population studies in humans and functional studies in animal models have shown that Angptl3 and Angptl4 are potent regulators of triacylglycerol metabolism but their mechanism of action is not completely understood.

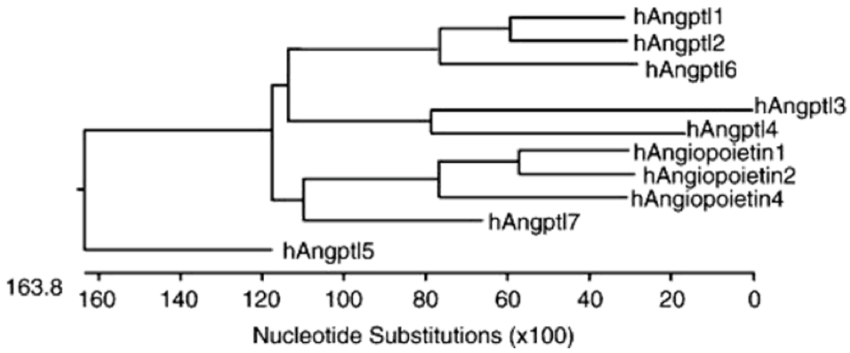

Figure 9. **Phylogenetic trees for Angptls and angiopoietins (Angpt) (Hato et al, 2008).** (Reprinted with permission from Elsevier).

2.2.1 Angiopoietin-like 3

Conklin and collaborators discovered Angptl3 in 1999 while searching the expressed sequence tags databases for signal sequences and amphipathic helices (Conklin et al, 1999). Mouse *Angptl3* gene is located on chromosome 4 spanning 9404 bp, with 7 exons encoding a 455 amino acids secreted protein. The human *ANGPTL3* gene is located on chromosome 1 (1p31.1-p22.3) spanning 7994 bp and encodes a 460 AA protein.

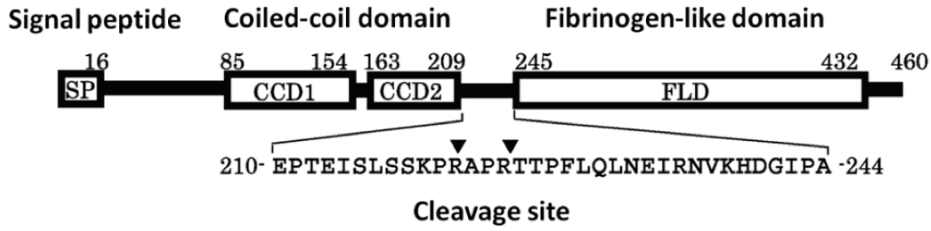

Figure 10. Schematic representation of Angptl3 modular structure (Ono et al, 2003). (Reprinted with permission from American Society for Biochemistry and Molecular Biology)

2.2.1.1 Transcriptional regulation

ANGPTL3 is expressed almost exclusively in the liver, with minor expression found in the kidney (Romeo et al, 2009). An important regulator of Angptl3 expression is liver X receptor (LXR), a nuclear receptor activated by oxysterols that control whole body cholesterol and lipid homoeostasis (Kaplan et al, 2003). LXR response element is located within the promoter of ANGPTL3 and it is likely that some of the effects of LXR on lipid metabolism are mediated by Angptl3 (Inaba et al, 2003). Several other molecules have been implicated in the control of ANGPTL3 gene expression, e.g. peroxisome proliferator-activated receptor delta (PPAR) δ, insulin, leptin, thyroid hormone and lipopolysaccharide (Fugier et al, 2006; Lu et al, 2010; Matsusue et al, 2006; Shimamura et al, 2004).

2.2.1.2 Post-translational modifications

Angptl3 undergoes several post-translational modifications such as oligomerisation, cleavage and glycosylation. Angptl3 forms oligomers, a feature that is shared with angiopoietins (Figure 8), but the importance of the modification for protein function or turnover is currently not known. Proprotein convertases cleave Angptl3 at two sites in the linker region, Arg^{221}/Ala^{222} and Arg^{224}/Thr^{225}, to generate the CCD and FLD and to activate its function (Figure 10) (Ono et al, 2003). Interestingly, Angptl3 is *O*-glycosylated at Thr^{226} by UDP-GalNAc: polypeptide N-acetylgalactosaminyltransferase 2 (GalNAc-T2) modification that reduces Angptl3 cleavage and possibly its function (Ono et al, 2003; Schjoldager et al, 2010). Variants in the *GALNT2* gene are associated with increased triacylglycerol levels in genome wide association studies (GWAS) possibly due to altered GalNac-T2-mediated *O*-glycosylation of Angptl3 (Hegele et al, 2009; Schjoldager et al, 2010; Teslovich et al, 2010). Lack of GalNac-T2 would be expected to reduce *O*-

glycosylation and increase the cleavage of Angptl3, which inhibits the LPL activity, and thus, increases triacylglycerol levels. This interesting possibility was not recently confirmed in carriers of *GALNT2* loss-of-function mutations where reduced plasma triacylglycerols levels and no difference in Angptl3 processing were observed (Holleboom et al, 2011).

2.2.1.3 Inhibition of lipoprotein lipase

The best understood function of Angptl3 is regulation of plasma triacylglycerol levels mainly by LPL inhibition. This was first demonstrated in KK/San mice that have low plasma triacylglycerol levels due to a 4-bp insert in exon 6 of the *Angptl3* gene, creating a stop codon at position 347 (Koishi et al, 2002). Koshi and colleagues demonstrated that overexpression of Angptl3 or intravenous injection of the purified protein in KK/San or C57BL/6 J mice elicited an increase in plasma triacylglycerol levels (Koishi et al, 2002). Same group demonstrated further that the phenotype in KK/San mice is mainly due to enhanced clearance of VLDL with small to no differences in hepatic VLDL secretion rate (Shimizugawa et al, 2002). Furthermore, *in vitro* analysis revealed that Angptl3 directly inhibits LPL activity in a concentration dependent manner (Shimizugawa et al, 2002) whereas *in vivo* targeted disruption of Angptl3 increased post heparin plasma LPL activity (Koster et al, 2005). The increase in plasma triacylglycerol levels by Angptl3 is most prominent during the fed state, suggesting that Angptl3 mainly plays a role in regulation of peripheral lipid uptake after feeding (Koster et al, 2005). In contrast to *in vivo* effect, Angptl3 inhibits LPL activity weakly and reversibly *in vitro* possibly because of Angptl3-induced cleavage of LPL (Liu et al, 2010; Shan et al, 2009). Cell-based analysis showed that Angptl3 enhances the cleavage of LPL by paired amino acid converting enzyme-4 (PACE4) and furin causing dissociation of LPL from the cell surface (Liu et al, 2010). It appears that HSPG and GPIHBP1 protect LPL activity from Angptl3 inhibition but not from Angptl3-induced LPL cleavage (Liu et al, 2010; Shan et al, 2009; Sonnenburg et al, 2009).

2.2.1.4 Human genetic studies

Genome wide association studies (GWAS) in humans have shown that single nucleotide polymorphisms (SNPs) at loci near the *ANGPTL3* are associated with plasma concentration of triacylglycerol (Kathiresan et al, 2008; Teslovich et al, 2010; Willer et al, 2008). One *ANGPTL3* SNP identified in GWAS, rs12130333, is also associated with the Fredrickson hyperlipoproteinemia type 5, representing a mixed hyperlipidaemia characterized by elevated VLDL and chylomicron levels (Hegele et al, 2009). Re-sequencing of the seven exons of *ANGPTL3* in the Dallas Heart Study

showed further evidence for a link between the *ANGPTL3* gene and plasma triacylglycerol levels in humans (Romeo et al, 2009). All sequences predicted to be loss-of-function alleles (frameshift 122 [Fs122], FsQ192, and FsK455) were identified in the lowest quartile of triacylglycerol levels, suggesting that deficiency in Angptl3 is associated with reduced triacylglycerol levels in plasma (Romeo et al, 2009). Furthermore, Musunuru et al. identified E128X and S17X as two novel nonsense mutations in *ANGPTL3* by sequencing the exome of two individuals with familial combined hypolipidemia, a phenotype characterized by a dramatic reduction in all major plasma lipoproteins (Musunuru et al, 2010). Lipoprotein metabolic studies in humans suggested that Angptl3 deficiency decreases VLDL production rate, contributing to the low triacylglycerol levels in plasma (Musunuru et al, 2010). Thus, human genetic studies clearly link the *ANGPTL3* gene to regulation of plasma lipid levels, with a primary effect on plasma triacylglycerol.

2.2.1.5 Mobilization of triacylglycerol stores

Angptl3 deficiency in mice was shown to decrease plasma free fatty acids whereas injection of recombinant or adenoviral overexpression of Angptl3 robustly increased plasma free fatty acids (Koishi et al, 2002). This effect is explained by the increased in intracellular adipose tissue lipolysis (Shimamura et al, 2003). Indeed, Angptl3 binds to adipocyte surface and triggers the release of free fatty acids. Interaction with the surface of adipocyte was dependent on HSPGs, as the addition of soluble heparin greatly reduced the Angptl3 binding (Shimamura et al, 2003). If the same would happen in humans, it provides a mechanism for the decreased VLDL production rate observed in familial combined hypolipidemia (Musunuru et al, 2010). A decrease flux of free fatty acids from adipose tissue to the liver, due to Angptl3 deficiency, is expected to decrease the VLDL secretion (Gibbons et al, 2000; Taskinen, 2003). Therefore, Angptl3 is potentially an important factor in the crosstalk between adipose tissue and liver to regulate whole body triacylglycerol homeostasis.

2.2.1.6 Other functions

In addition to its role as LPL inhibitor, Angptl3 is able to inhibit endothelial lipase (EL), an enzyme involved in high density lipoprotein (HDL) catabolism, therefore plasma Angptl3 levels are positively correlated with HDL cholesterol and phospholipid levels (Shimamura et al, 2007). The suggested mechanism involves activating cleavage of Angptl3 by hepatic proprotein convertases (Jin et al, 2007). Angptl3 cleavage and function was shown to be inhibited both *in vitro* and *in vivo* by profurin, an intracellular inhibitor of proprotein convertases (Jin et al, 2007).

If the Angptl3-CCD is implicated in the inhibition of LPL and EL, the FLD gives Angptl3 proangiogenic properties (Camenisch et al, 2002). Angptl3-FLD was shown to bind alpha(v)beta(3) integrin and to induce angiogenesis in vivo (Camenisch et al, 2002).

2.2.2 Angiopoietin-like 4

Kim et al. identified Angptl4 using degenerate PCR of a human embryonic cDNA to obtain a product with a novel sequence related to the angiopoietins (Kim et al, 2000). Concurrently, Angptl4 was isolated and characterized as a novel target for the nuclear receptors PPARγ and PPARα (Kersten et al, 2000; Yoon et al, 2000).
Mouse *Angptl4* is located on chromosome 17 and has 7 exons encoding for a 410 amino acids protein. The human *ANGPTL4* is located on chromosome 19 (19p13.3), has a similar organisation with mouse *Angptl4* and encodes a 406 amino acid protein.

2.2.2.1 Transcriptional regulation

In contrast to Angptl3, Angptl4 is ubiquitously expressed but the tissue with highest expression for humans is liver and for mice the adipose tissue (Kersten et al, 2000; Romeo et al, 2009). As it was originally discovered, the *ANGPTL4* gene is a target of PPARα and -γ in liver and adipose tissue, respectively (Kersten et al, 2000; Yoon et al, 2000). In other tissues like skeletal and cardiac muscle, PPARδ is a main regulator of Angptl4 expression (Georgiadi et al, 2010; Staiger et al, 2009). Accordingly, *ANGPTL4* is the most highly induced gene by fatty acids, activators of PPARs, in several cell types (Georgiadi et al, 2010; Mattijssen & Kersten, 2011; Staiger et al, 2009). Other stimuli such as transforming growth factor β (TGFβ), insulin, hypoxia or glucocorticoids can also control Angptl4 expression in various cell types (Koliwad et al, 2009; Le Jan et al, 2003; Padua et al, 2008; Yamada et al, 2006).

2.2.2.2 Post-translational modifications

Angptl4 is cleaved at the conserved proprotein convertase recognition sequence ^{161}RRKR^{164} to generate an N-terminal CCD and a C-terminal FLD (Figure 11) (Ge et al, 2004a; Yin et al, 2009). Proprotein convertases (PC), such as furin, PC5/6, PACE4, and PC7, are all able to cleave human Angptl4 at a consensus site but whether this is important for Angptl4 function is not clear (Lei et al, 2011; Yin et al, 2009). Angptl4 forms oligomers intracellularly, before secretion, in a process dependent on disulfide bonds (Ge et al, 2004a; Yin et al, 2009). Since oligomerisation is a property given by the CCD, both full length and N-terminal fragment can be

detected as oligomers secreted by the cells under nonreducing gel electrophoresis (Ge et al, 2004a; Yin et al, 2009). In addition, N-glycosylation of FLD was suggested to play an important role in Angptl4 angiogenic potential whereas sialylation of Angptl4 impact the glomerular ultrafiltration (Clement et al, 2010; Yang et al, 2008).

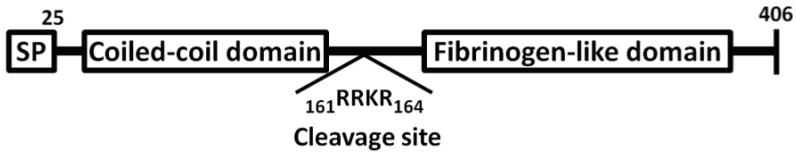

Figure 11. Schematic representation of Angptl4 modular structure

2.2.2.3 Inhibition of lipoprotein lipase

The first hint about Angptl4 function was provided by Yoshida et al. in 2002. They reported an increase in plasma lipid levels after intravenous injection of the Angptl4 protein in KK/San mice, as well as an inhibition of LPL activity *in vitro* (Yoshida et al, 2002). Further studies provided convincing evidence that Angptl4 increases plasma triacylglycerol levels in mice by inhibiting LPL activity (Ge et al, 2004b; Koster et al, 2005; Xu et al, 2005; Yu et al, 2005). The mechanism by which Angptl4 inhibits LPL activity proved to be very interesting. The CCD of Angptl4 inhibits LPL by promoting the conversion of catalytically active LPL dimers into catalytically inactive LPL monomers, thereby permanently inactivating LPL (Sukonina et al, 2006). In mice, injection of monoclonal antibody directed against the CCD of Angptl4 lowers plasma TG to levels comparable to Angptl4−/− animals (Desai et al, 2007). Further studies established that the CCD of Angptl4 irreversibly inhibits LPL activity at least partially by generation of inactive monomers (Shan et al, 2009; Yau et al, 2009). Recently, it was reported that Angptl4 CCD stimulates LPL proteolysis by cell associated proprotein (Lei et al, 2011). It is tempting to speculate that the latter mechanism is secondary to the generation of inactive monomers. The LPL monomers have lower affinity to heparin and might be more susceptible to proteolysis, as it is the case with Angptl4 (Chomel et al, 2009).

2.2.2.4 Human genetic studies

Evidence for a role of Angptl4 in regulating plasma TG homeostasis in humans came together with a technological breakthrough as *ANGPTL4* was resequenced in a

large number of individuals (n = 3,551) (Romeo et al, 2007). One variant, E40K, was identified as present in approximately 3% of European Americans and associated with significantly lower plasma levels of triacylglycerol and higher levels of HDL (Romeo et al, 2007). The finding was replicated by other groups and interestingly, differences between E40K carriers and non-carriers persisted over a nine-year period during which triacylglycerol concentrations went up in both groups (Nettleton et al, 2008; Talmud et al, 2008). Surprisingly, these findings are not supported by the recent GWAS studies that associated *ANGPTL4* with HDL rather than triacylglycerol levels in plasma (Kathiresan et al, 2008; Teslovich et al, 2010).

2.2.2.5 Mobilization of triacylglycerol stores

Besides raising circulating triacylglycerol levels, Angptl4 potently increases intracellular adipose tissue lipolysis. Injection of recombinant Angptl4 in mice acutely raises free fatty acids, suggesting enhanced breakdown of triacylglycerols in adipose tissue (Yoshida et al, 2002). In accordance with this, Angptl4 overexpression augments the fasting-induced increase in free fatty acids, whereas this response is completely blunted in Angptl4−/− mice (Sanderson et al, 2009). Furthermore, very recent data established Angptl4 as a physiological mediator of intracellular lipolysis in murine adipocytes during fasting (Gray et al, 2012). Angptl4 was shown to be necessary for the ability of glucocorticoids and catecholamines to mobilize the fatty acids from triacylglycerol stores in adipocytes (Gray et al, 2012). This is achieved by Angptl4-dependent increase in adipose tissue cAMP levels and the phosphorylation of cAMP-dependent components of the lipolytic machinery, such as HSL and PLIN1 (Gray et al, 2012). As FLD and CCD mediate different Angptl4 functions, it will be interesting to know which is responsible for stimulation of intracellular lipolysis in adipocytes and if cleavage is an important regulatory step. As Angptl4 is expressed and secreted from most of the tissues, it remains to be seen if it is functioning in an endocrine or autocrine/paracrine manner. Further research is also necessary to determine if interaction with integrins and HSPGs play an important role in this process.

2.2.2.6 Other functions

In the recent years, Angptl4 has emerged as a multifunctional protein and important regulator of physiological and pathological processes in a variety of tissues. Angptl4 is expressed in the mouse hypothalamus, cortex and pituitary gland, and was suggested to function as an anorexigenic factor (Kim et al, 2010; Wiesner et al, 2004). Administration of Angptl4 lowered food intake and body weight gain possibly via inhibition of hypothalamic AMPK activity (Kim et al, 2010). It will be interesting to see how Angptl4 function in hypothalamus is coordinated with its activity in mobilizing triacylglycerol stores from adipose tissue (Gray et al, 2012; Mandard et al, 2006). Moreover, fibrinogen-like domain of Angptl4 was shown to interact and activate specific integrins in order to modulate wound healing, angiogenesis, vascular permeability and tumorigenesis (Goh et al, 2010; Le Jan et al, 2003; Padua et al, 2008; Zhu et al, 2011).

3 AIMS OF THE STUDY

The aim of this work was to understand the roles of Angptl3 and Angptl4 in the regulation of human energy and lipoprotein metabolism and their impact on obesity and cardiovascular diseases using population samples and cell biological approach.

Specific goals of the thesis were:

1. to develop and validate quantitative methods for the measurement of Angptl3 and Angptl4 in humans;
2. to determine the correlations between Angptl3 and Angptl4 in human plasma with risk factors for cardiovascular diseases and obesity;
3. to determine the mechanisms for tissue specific regulation and function of Angptl4.

4 MATERIALS AND METHODS

4.1 Published methods

List of published methods

Method	Original publication
AAV gene delivery *in vivo* and *in vitro*	III
Affinity chromatography	I
ELISA	I, II, III, IV
Fatty acid oxidation	III
Fluorescence microscopy	III
Glucose uptake	III
Glycogen synthesis	III
LPL activity assay	III
Microarray analysis	IV
Oil Red O staining	III
Plasma lipid measurements	I, II, III, IV
Real time PCR	III
RNA extraction	III
RNA interference	III
RT-PCR	III
SDS-PAGE and Western blot	I, II, III
Statistical analyses	I, II, III, IV
Transient transfection	I, II, III

Health 2000 Health Examination Survey (original paper I)
Health 2000 Health Examination Survey was carried out in Finland from fall 2000 to spring 2001 (Aromaa & Koskinen, 2002). Serum samples from a random sub-sample consisting of 250 subjects (125 men and 125 women), age range 30 – 94 years was used to study the correlations between Angptl3 and Angptl4 and major plasma lipid levels.

Northwick Park Heart Study II (original paper II)
Northwick Park Heart Study II (NPHSII) is a population-based prospective cohort comprising of 3012 European Caucasian men aged 50-63 years recruited from nine general medical practices in the United Kingdom (Robertson et al, 2003). Individuals were excluded if they had pre-existing cardiovascular disease (coronary heart

disease or stroke), coronary surgery or malignant disease, or were taking Aspirin or anticoagulant. Subjects were followed for the development of coronary heart disease from 1989. Six SNPs were used to genotype NPHSII: rs4076317 (-207C>G), rs7255436 (3991A>C), rs1044250 (6959C>T, T266M), rs11672433 (9511A>G), rs7252574 (12574C>T), and rs1808536 (12651G>A), in addition to E40K (118G>A) (Talmud et al, 2008). These SNPs captured >92% of the genetic variation in *ANGPTL4*. Genotyping in NPHSII was performed using TaqMan technology (Applied Biosciences, ABI, Warrington UK) as previously reported (Talmud et al, 2008). Based on their T266M genotype, 666 subjects were selected to study the relationship between Angptl4 levels and plasma triglycerides.

FinnTwin12 and FinnTwin16 studies (original paper IV)

For this study, 121 twin pairs (46 monozygotic and 75 dizygotic) were selected from two population-based longitudinal studies, FinnTwin16 (FT16) and FinnTwin12 (FT12) each consisting of five consecutive birth cohorts of Finnish twins (n=5601 subjects in FT16 and n=5184 in FT12) (Kaprio, 2006). Of the 121 pairs studied, 21 monozygotic pairs and 48 dizygotic pairs were discordant for obesity with intra-pair difference in BMI $\geq$ 2.5 kg/m^2, and one twin obese (BMI $\geq$ 30) and the other co-twin non-obese. Their weight had been stable for at least three months prior to the study. Weight and height were measured after 12-hour overnight fast barefoot and in light clothing to calculate BMI. BMI $\geq$25 kg/m^2 was used as a cut-off for overweight and BMI $\geq$30 kg/m^2 as a cut-off for obesity. Body composition was measured using whole body DXA scans. Serum Angptl4 concentration and adipose tissue *ANGPTL4* expression of were studied in relationship with obesity-related parameters.

Statistical analyses

SPSS version 16.0 or 17.0 for Windows (SPSS, Inc.) and GraphPad Prism 4.03 (GraphPad Software, Inc.) were used to perform the statistical analyses. Parameters were logarithmically transformed before Pearson correlations tests were performed. In the Twins study, Pearson correlations were corrected for clustered sampling of co-twins and within-pair differences were calculated to control for genetic influences. In addition, Spearman test, partial correlations, multivariate linear regression analyses, Student t-test and Mann Whitney test were employed in this work.

Enzyme-linked immunosorbent assay

Noncompetitive ELISAs were developed to determine the concentration of human serum/plasma Angptl3 and Angptl4. A rabbit polyclonal antibody, raised against recombinant Angptl3 (aa 17-223) (BioVendor, Czech Republic), was used as capture antibody whereas a biotinylated sheep IgG, raised against human recombinant Angptl3 (aa 17-460) (RnD Systems, Minneapolis, USA), was used for detection. Antibodies from the DuoSet Elisa hAngptl4 (RnD Systems, Minneapolis, USA)

were used to capture and detect Angptl4. Plates with 96 wells were coated with the capture antibodies, blocked and incubated with samples and standards. After the incubation with detection antibodies and streptavidin-HRP, the colorimetric reaction was performed and plates analyzed at 450 nm. A standardized human serum (for Angptl3) or recombinant protein (for Angptl4) was used to construct the standard curves. A second order polynomial equation was used to fit the standards and calculate the sample concentrations.

Microarray analysis

Subcutaneous adipose tissue biopsies were collected under local anesthesia using a surgical technique. Total RNA was extracted and the quality was analyzed using the 2100 Bioanalyzer platform (Agilent Technologies). Adipose tissue transcriptomics was obtained using the Affymetrix U133 Plus 2.0 chips. Expression values were normalized and analyzed using the GeneSpring GX 7.3 software (Agilent Technologies).

Lipoprotein lipase activity

LPL activity was measured as previously described (Huttunen et al, 1975). LPL substrate was obtained using [Carboxyl-14C]-Triolein (S.A. 2.2 GBq/mmol, PerkinElmer) and glyceryl trioleate emulsified in the presence of gum arabic as previously described. Samples were incubated with the substrate and human serum (as a source for apoC-II, LPL cofactor) for 1 hour at 37^0C. Reaction was stopped by addition of 3.25 ml of methanol-chloroform-heptane (1.41:1.25:1.00, vol/vol/vol) and 0.75 ml of potassium carbonate/borate buffer (pH 10.5). Hydrophilic phase (BSA-FAs) and hydrophobic phase (triolein) were separated by centrifugation and radioactivity was measured from both fractions by liquid scintillation counting (Wallac LS Counter, Turku, Finland). Activity was expressed as mol of FAs released per hour per mg of cell protein.

Fatty acid oxidation

Fatty acid oxidation was measured using [1-^{14}C]-Palmitic acid (S.A. 2 GBq/mmol, ARC 0172A, ARC) or [9,10-^{3}H(N)]-Palmitic acid (S.A. 1.5 TBq/mmol, ART 0129, ARC). The oxidation of palmitic acid was analysed by measuring the formation of ^{14}C-CO_2, ^{3}H-H2O and ^{14}C-acids soluble metabolites (Hirschey & Verdin, 2010; Rune et al, 2009). Radioactivity was measured by liquid scintillation counting (Wallac LS Counter, Turku, Finland) and values expressed as µmol of FA oxidized per minute per mg of cell protein.

Primary human myotubes
Muscle samples for primary human myoblast cultures were obtained from surgical open muscle biopsies. Biopsies taken from 6 nondiabetic men were minced and digested with collagenase and trypsin. Satellite cells were isolated, and myoblasts proliferated and differentiated for seven days in low serum medium (Al-Khalili et al, 2003).

Other methods
Standard methodology such as cell culture, AAV gene delivery, fluorescence microscopy, Oil Red O staining, lipid measurements, real time PCR, RNA extraction, RNA interference, RT-PCR, SDS-PAGE and Western blot, transient transfection have been applied in this thesis as described in the original publications.

4.2 Unpublished methods

Adipose tissue lipolysis
Lipolysis was measured as the rate of glycerol release. In brief, Simpson-Golabi-Behmel syndrome (SGBS) adipocytes in 12-well plates were incubated in 0.5 ml of Krebs-Ringer HEPES (KRH) buffer with 1% fat free BSA in the presence of 1 µg/ml recombinant human Angptl4 or 100nM isoproterenol or left untreated. Aliquots of the KRH buffer were collected at two and four hours and the glycerol concentrations were determined by using a fluorimetric assay kit (BioVision). Cell lysates were analysed for protein and intracellular triacylglycerol concentrations to normalize the lipolytic signals. Intracellular triacyglycerol concentration was measured by an enzymatic colorimetric assay (Cobas, Roche/Hitachi).

Campodimele population study
In Campodimele, a small town located in the province of Latina (Italy), were previously identified subjects with reduced plasma levels of LDL-cholesterol (LDL-C) and ApoB without mutations in apolipoprotein B gene (Fazio et al, 1991). Analysis of the *ANGPTL3* by re-sequencing revealed the presence of S17X loss of function mutation in a total of 55 heterozygotes and 8 homozygotes. Plasma lipids were assayed as previously reported (Campagna et al, 2002). Angptl3 serum levels were determined using an ELISA (described in the original publication I) in *ANGPTL3* mutation carriers and in 49 age, gender and BMI-matched non-carriers. Serum samples were diluted 50-fold and measured in duplicate. Repeated measurements were made in homozygotes after 10-fold dilution of serum samples. Post heparin plasma was collected 15 minutes after a heparin injection (100 IU/kg body weight).

Lipoprotein profile by Fast Phase Liquid Chromatography (FPLC).
Serum aliquots of 100 µl were applied on a Superose 6HR gel filtration column (Pharmacia Biotech, Uppsala, Sweden) at a flow rate of 0.3 ml/min in PBS, pH 7.4 containing 1mM EDTA. During chromatography fractions (0.6 ml) were collected and analysed for cholesterol and triglyceride concentrations using enzymatic colorimetric assays (Cobas, Roche/Hitachi).

5 RESULTS AND DISCUSSION

5.1 Angiopoietin-like 3

5.1.1 Relationship between Angptl3 levels and lipids in plasma

Extensive data derived from mouse studies, *in vitro* biochemical analyses and human genetics clearly defined Angptl3 as a modulator of plasma triglyceride levels via LPL inhibition (Kersten, 2009). Based on these findings, plasma Angptl3 levels are expected to positively correlate with plasma triacylglycerol levels. Analysis of a Japanese population suggested that the Angptl3 levels in circulation are not correlated with plasma triacylglycerol levels (Hatsuda et al, 2007). Two other studies measured plasma Angptl3 levels and although no correlation with triacylglycerol levels has been found the populations were too small to provide convincing evidence (Nakajima et al, 2010; Shimamura et al, 2007). To clarify this intriguing Angptl3-triacylglycerol relationship an in house ELISA was developed using high quality affinity purified antibodies (original publication **I**). Angptl3 ELISA was specific for human Angptl3 with detection limit of 1 ng/ml and intra- and inter-assay coefficients of variation less than 10 %. The in house ELISA was compared with a commercially available kit and Angptl3 levels obtained in 18 subjects were highly correlated, $p < 0.0001$ (original publication **I** and Figure 12).

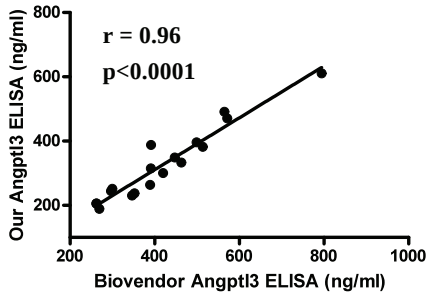

Figure 12. Correlation between in house Angptl3 ELISA and a commercially available kit from BioVendor (n=18) (unpublished data).

Serum Angptl3 levels in a random subsample of the Health 2000 Health Examination Survey (n= 250 subjects) demonstrated high inter-individual variability with a distribution skewed to the left, but normalised after logarithmic transformation (Figure 13). Linear regression analysis revealed a negative correlation between serum Angptl3 and fasting triacylglycerols levels (Pearson correlation, $r = -0.182$, $p = 0.004$). This is the opposite what would be expected from the inhibitory effect of Angptl3 on LPL activity. Interestingly, this correlation was lost when HDL cholesterol (HDL-C) and apoA-I levels were used as control variables (partial correlation, $r = -0.029$, $p = 0.649$) (original publication **I**). Therefore, the correlation with fasting triglycerides might be dependent on Angptl3 effect on HDL-C levels. As Angptl3 was shown to inhibit also endothelial lipase (EL), it is not surprising that a strong correlation with plasma HDL-C levels was found (Pearson correlation, $r = 0.224$, $p < 0.001$) (Jin et al, 2007; Shimamura et al, 2007). These data suggest that plasma Angptl3 is a more potent EL inhibitor than LPL inhibitor in humans or that different mechanisms of action towards the two lipases is responsible for the observed correlations.

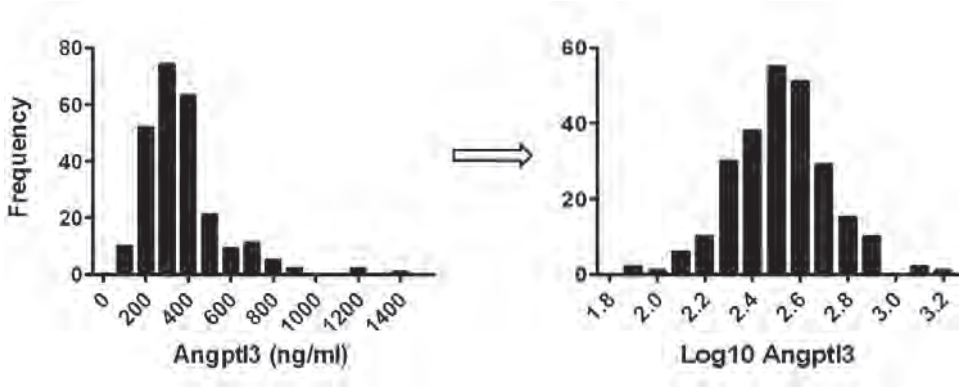

Figure 13. Angptl3 distribution in a normal Finnish population before (left) and after (right) logarithm transformation (unpublished data).

Elevated plasma levels of triacylglycerol and reduced HDL-C are important components of the metabolic syndrome, a cluster of risk factors for CVD and type 2 diabetes mellitus (Alberti et al, 2009). Therefore, Angptl3 is a possible target for the development of new therapeutic strategies to reduce mortality associated with CVD and type 2 diabetes mellitus.

5.1.2 Angptl3 deficiency and familial combined hypolipidemia (unpublished data)

Further knowledge about the role of Angptl3 in the plasma triacylglycerol metabolism in humans was obtained by studying the familial combined hypolipidemia.
Elias and colleagues have reported an interesting form of hypobetalipoproteinemia, very low plasma apoB levels, not linked to the *APOB* gene where some of the affected subjects also had very low HDL-C levels (Elias et al, 2000). A decade later, Musunuru and collaborators revealed the mystery behind this phenotype using exome sequencing (Musunuru et al, 2010). Analysis of two subjects with very low apoB and HDL levels revealed two causal non-sense mutations in the first exon of *ANGPTL3*, E129X and S17X. The authors proposed to call this phenotype familial combined hypolipidemia (Musunuru et al, 2010). S17X mutation and the familial combined hypolipidemia are highly prevalent in a small town of Campodimele (Latina, Italy) providing a great opportunity to study Angptl3 function in detail (Fazio et al, 1991). Consistent with the S17X variant being a null allele, serum Angptl3 was undetectable in homozygous carriers whereas in heterozygotes showed a 42% reduction compared to non carriers ($p<0.0001$) (Figure 14).

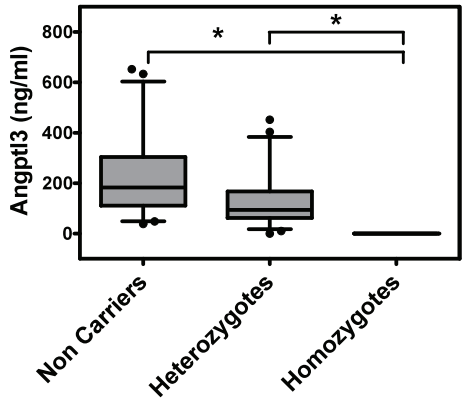

Figure 14. **Serum Angptl3 levels according to the number of mutant *ANGPTL3* alleles (unpublished data)**. Shown are the levels of angiopoietin-like 3 (ANGPTL3) protein according to the *ANGPTL3* genotype. The box plots represent the 25% and 75% percentile and whiskers represent the 5-95 percentiles (delineated by the top and bottom of each box). Outliers falling below the 5th percentile or above the 95th percentile are represented by points below or above the whiskers, respectively. The median levels are shown by the middle horizontal line in each box. *$p<0.05$, Kruskal-Wallis test and post test analysis using Dunn's Multiple Comparison Test.

Consistent with the data obtained in the Health 2000 study (original publication **I**), a reduction in Angptl3 concentration in heterozygotes translated in a significant decrease in HDL-C levels (Table 1). S17X homozygotes showed the phenotype characteristic for familial combined hypolipidemia, with major plasma lipids dramatically reduced (Table 1).

Table 1. Comparison of clinical characteristics and plasma lipids in carriers and non-carriers of mutations in the *ANGPTL3* gene (unpublished data)[1]

	Age years	Gender (M/F)	BMI	TC mmol/L	LDL-C mmol/L	HDL-C mmol/L	TG mmol/L
Non carriers (n=339)	53 ± 20	161/180	28 ± 5	4.7 ± 0.8	2.6 ± 0.8	1.6 ± 0.4	1 ± 0.6
Heterozygotes (n=55)	50 ± 20	24/31	29 ± 5	4.4 ± 0.8§	2.5± 0.7	1.4 ± 0.4§	0.9 ± 0.5
Homozygotes (n=8)	65 ± 14	4/4	29 ± 6	2.4 ± 0.3*	1.3 ± 0.4*	0.8 ± 0.2*	0.4 ± 0.1*

[1]Differences between variables were evaluated using Mann-Whitney test. *p< 0.01 for comparison between homozygous carriers vs. non-carriers or heterozygous. § p<0.05, for comparison between heterozygous carriers vs. non-carriers.

This was further supported by analyzing the plasma lipoprotein profile by Fast Performance Liquid Chromatography (FPLC) in S17X homozygotes and non-carriers (Figure 15). These data demonstrate that familial combined hypolipidemia segregates as a recessive trait with plasma lipoproteins comprehensively affected only by the total deficiency of Angptl3.

To gain further knowledge about the mechanism by which Angptl3 affects plasma lipids levels in humans LPL and hepatic lipase (HL) activities were measured in post heparin plasma (PHP). Interestingly, PHP-LPL activity was not different in heterozygotes compared with non carriers although a significant reduction in plasma Angptl3 levels is observed (Figure 16, left panel). Moreover, PHP-LPL activity was the same in the lowest Angptl3 quintile (median levels, 42 ng/ml) compared with the highest Angptl3 quintile (median levels, 307 ng/ml) (Figure 17). In contrast, in homozygotes the PHP-LPL activity was 3.5-fold higher compared with non carriers or heterozygotes (Figure 17, left panel). These data suggest that Angptl3 is a very potent LPL inhibitor but does not act in a concentration dependent manner *in vivo*, which is in contrast to *in vitro* data (Shimizugawa et al, 2002). It can be speculated that Angptl3 proteolysis rather than plasma Angptl3 levels are important

in regulating LPL activity and plasma triacylglycerol levels. A limitation of the ELISA used is that it is not known what form of Angptl3 is detected in plasma, full length, fragments or all of them.

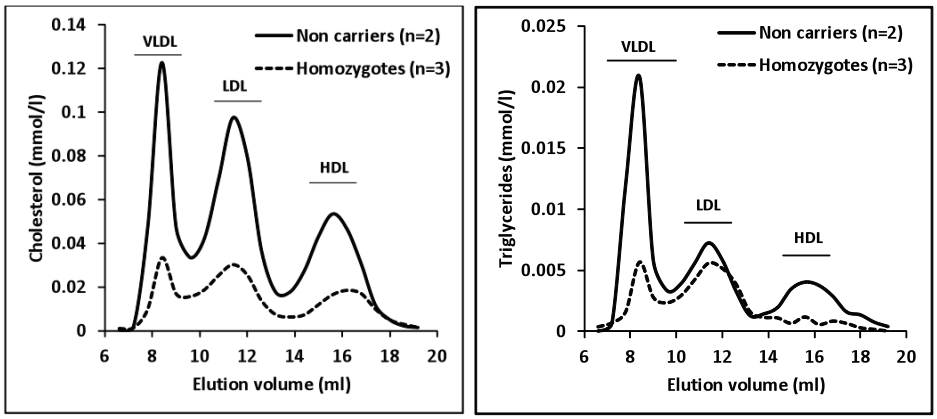

Figure 15. **Serum lipoprotein profiles for non-carriers versus homozygous carriers of S17X mutation (unpublished data).** Average Fast Phase Liquid Chromatography (FPLC) elution profiles for non-carriers (n=2) and for homozygotes (n=3) as assessed by cholesterol (Panel A) and triglycerides (Panel B) in elution fractions. Elution positions for VLDL, LDL and HDL are depicted**.**

Hepatic lipase (HL) displays both triglyceride and phospholipase activity and enhances the LDL and HDL catabolism (Olivecrona & Olivecrona, 2010). Angptl3 was shown to inhibit also HL activity therefore providing mechanistic insights for the dramatic reduction in LDL and HDL cholesterol levels observed in Angptl3 deficiency (Ando et al, 2003; Shimizugawa et al, 2002). However, PHP-HL activity was not changed according to the *ANGPTL3* genotype, suggesting that in contrast to mice, Angptl3 does not inhibit HL activity in humans (Figure 16, right panel).

The HDL cholesterol phenotype in the Campodimele population confirmed the previous observations that Angptl3 and HDL cholesterol levels are positively correlated, most likely by inhibiting the EL activity ((Table 1 and original publication **I**) and (Shimamura et al, 2007)).

If the changes in plasma triacylglycerol and HDL cholesterol levels can be explained by the inhibition of LPL and EL lipase activity, the reduction in LDL cholesterol levels remains a complete mystery. One possibility is that Angptl3 acts directly in the liver to regulate the LDL uptake (Musunuru et al, 2010).

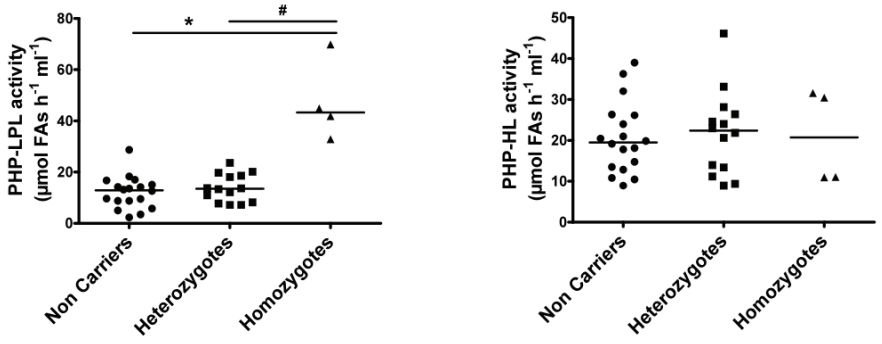

Figure 16. **Post heparin (PHP) lipoprotein lipase (LPL) activity (left panel) and PHP hepatic lipase (HL) activity (right panel) according to the *ANGPTL3* genotype (unpublished data).** * $p < 0.05$, Mann-Whitney test**.**

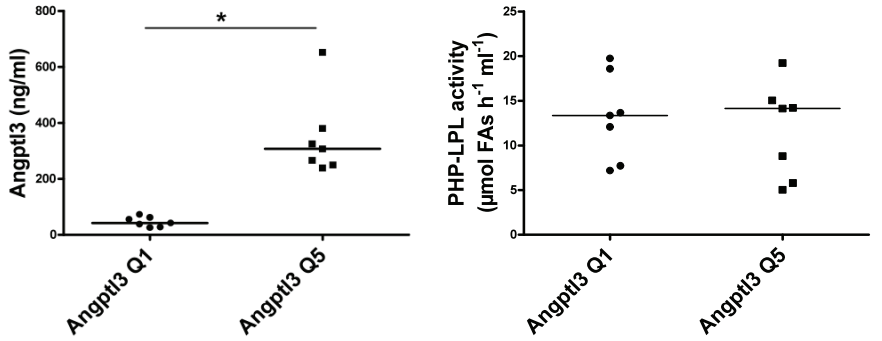

Figure 17. **Angptl3 levels in plasma (left panel) and post heparin (PHP) lipoprotein lipase (LPL) activity (right panel) in the lowest (Q1) and highest (Q5) quintiles of plasma Angptl3 (unpublished data).** * $p < 0.05$, Mann-Whitney test**.**

5.2 Angiopoietin-like 4

5.2.1 Relationship between Angptl4 levels and lipids in plasma

Data obtained using mouse models and *in vitro* biochemical analyses provided strong evidence that Angptl4 can act as a modulator of plasma triglyceride levels via LPL inhibition (Kersten, 2009). Although GWAS studies did not associate *ANGPTL4* with plasma triacylglycerol, several studies showed that the deleterious non-synonymous mutation, E40K, is associated with low plasma triacylglycerol levels (Mattijssen & Kersten, 2011). Two small studies utilizing the same commercial ELISA provided contradictory results for the correlation of plasma Angptl4 and triacylglycerol levels (Staiger et al, 2009; Stejskal et al, 2008). To clarify the relationship between the two parameters in humans an in house ELISA for Angptl4 has been developed and validated using a previously described protocol based on a commercially available antibody (Kersten et al, 2009). The detection limit of the assay was 0.1 ng/ml for Angptl4 and the intra- and inter-assay coefficients of variation were less than 10 % (original publication **I**). No cross-reactivity was observed with human recombinant Angptl3 or with sera derived from different mammalian species, mouse, rabbit and bovine. Dilution linearity showed a coefficient of variation of 9.6 % and the recovery of recombinant protein added to serum was on average 104 % (original publication **I**).

The Angptl4 ELISA was utilised to measure serum Angptl4 levels in a random subsample of the Health 2000 Health Examination Survey (n= 250 subjects). Angptl4 levels demonstrated high inter-individual variability with a distribution skewed to the left and partially normalised after logarithmic transformation (Figure 18).

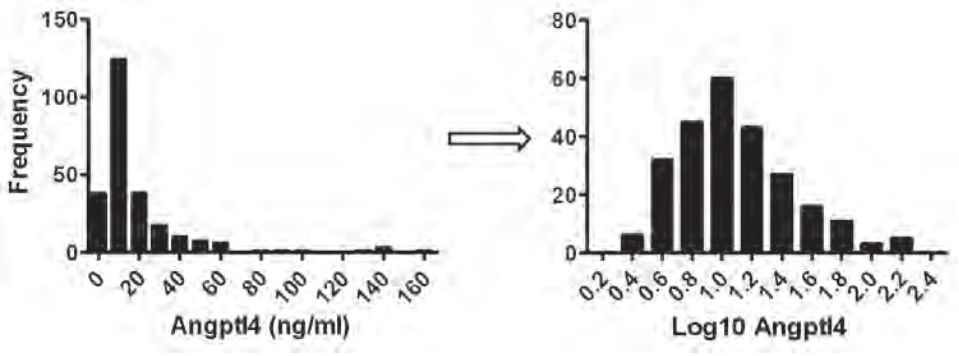

Figure 18. Angptl4 distribution in a normal Finnish population before (left) and after (right) logarithm transformation (unpublished data).

Serum Angptl4 levels were positively correlated with free fatty acids ($p = 0.044$) in accordance with the induction of Angptl4 synthesis via PPARs (Kersten et al, 2009). Interestingly there was no correlation between serum Angptl4 and fasting triacylglycerol levels as well as other lipid parameters investigated (original publication **I**). Because Angptl4 levels could not be fully normalised and had a very high inter-individual variation it might be that this study was too small to firmly establish the lipid parameters related to Angptl4 in humans. Therefore a larger study in which Angptl4 levels were analysed together with seven *ANGPTL4* SNPs was performed in 666 subjects of the Northwick Park Heart Study II (original publication **II**). Again, no correlation with triacylglycerol levels was observed but a negative correlation with plasma high-density lipoprotein cholesterol became evident ($p<0.01$) (original publication **II**, Table 2). The main SNP independently associated with higher Angptl4 levels was T266M ($p<0.001$) possibly by influencing Angptl4 secretion (original publication **II**, Figure 2). This provided a great opportunity to evaluate the relationship between Angptl4 levels, indirectly using T266M variant, and coronary heart disease risk in a very large population. A meta-analysis of 5 studies, with more than 4000 cases and 15 000 controls, demonstrated no association of the T266M with coronary heart disease risk (original publication **II**, Figure 3). This result was not affected by adjustment for age, gender, and plasma triacylglycerol and HDL-C levels.

All together these data suggest that Angptl4 in circulation is not a relevant modulator of plasma triacylglycerol metabolism in humans, rising questions on the mechanism by which Angptl4 inhibits LPL activity. There are several explanations for the results obtained. Using Angptl3 as an example, it is conceivable that in humans the Angptl4 effect on plasma triacylglycerol becomes evident only when non-functional Angptl4 is synthesised or when the protein is completely lacking in plasma (Romeo et al, 2007; Romeo et al, 2009). Another possibility is that Angptl4 could act at tissue level to fine tune LPL activity with the energy needs of the tissues. In contrast to Angptl3, Angptl4 is also expressed in tissues where LPL is synthesised and therefore it can act as a possible endogenous LPL inhibitor. Moreover, Angptl4 is a multifunctional protein and can have various effects acting as confounders in the population studies.

Since Angptl4 protein is cleaved after secretion it is therefore is important to know what the ELISA measures, as this could also affect the outcome of the studies. It has been previously shown that the antibody used in ELISA recognises full length Angptl4 in circulation (Kersten et al, 2009). Analysis of human serum could not provide convincing results (data not shown) but the study of purified Angptl4 isoforms and cell overexpressing Angptl4 demonstrated that the ELISA used quantifies mainly the C-terminal fragment (Figure 19). Although the C-terminal is not relevant

for LPL inhibition, the Angptl4 ELISA used in this thesis provided an accurate measurement of all Angptl4 isoforms as measured by western blot analysis (original publications **I**, **II** and **III**).

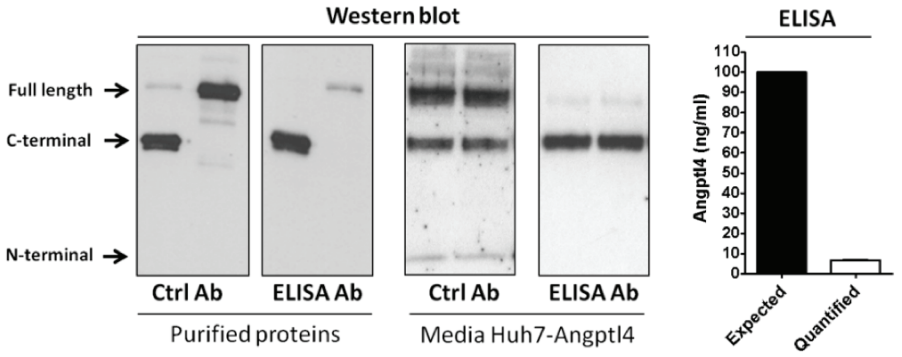

Figure 19. **ELISA affinity for different Angptl4 isoforms (unpublished data).** Western blot analysis was performed using 50 ng/lane of purified C-terminal and full length Angptl4 (Purified proteins) as well as using medium from Huh7 cells overexpressing Angptl4 (Media Huh7-Angptl4). Two antibodies were tested, a control antibody that recognise all Angptl4 isoforms (Ctrl Ab) and the antibody used in ELISA (ELISA Ab). Purified full length Angptl4 was quantified by ELISA and tested concentration (Expected) and actual values obtained by ELISA (Quantified) are presented**.**

5.2.2 Angptl4 as an endogenous LPL inhibitor

To study the capacity of endogenous Angptl4 to inhibit LPL activity an in vitro cell model of myotubes was employed. It is established that free fatty acids upregulate Angptl4 expression via PPARs and in myotubes Angptl4 is the most highly PPARδ-induced gene (Kersten, 2009; Staiger et al, 2009).

Studies using human and mouse myotubes showed that *ANGPTL4* gene and protein expression are increased up to two fold during differentiation of myoblasts into myotubes (original publication **III**, Figure 1 and Figure S1). These results suggest that Angptl4 might be part of the myogenic program and could play a role in the skeletal muscle energy metabolism.

Free fatty acids and a specific PPARδ agonist, GW501516, strongly upregulated ANGPTL4 gene and protein expression in myotubes ((Staiger et al, 2009) and (**IV**, Figure 2 and 3)). Western blot analyses showed that full-length, C-terminal, and N-terminal fragments of Angptl4 are secreted from myotubes after PPARδ activation (original publication **III**, Figure 3d). To evaluate if PPARδ activation can inhibit LPL activity, myotubes were incubated with GW501516 and heparin releasable LPL

activity was quantified. LPL activity was significantly inhibited after 24 hours incubation with GW501516, as compared to non treated cells (original publication **III**, Figure 4a). The GW501516 effect was fast, with a twofold reduction in LPL activity already after three hours of incubation (original publication **III**, Figure 4b). This suggests that PPARδ is involved in the rapid regulation of LPL activity at tissue level possibly to coordinate the tissue storage and oxidative capacity with the uptake of fatty acids (Muoio & Newgard, 2008; Olivecrona & Olivecrona, 2009). To provide further evidence for this, myotubes were incubated with Intralipid, a stable triglyceride emulsion and LPL substrate, in the presence or absence of GW501516. As expected, the uptake of fatty acids from Intralipid in myotubes was blocked in cells treated with PPARδ agonist (original publication **III**, Figure 4d and 4e). In contrast, the uptake of albumin bound free fatty acids was not affected by GW501516, demonstrating that the effect is dependent on LPL activity (original publication **III**, Figure 4d).

Overexpression of Angptl4 in myotubes resembles the GW501516 action and Angptl4 concentration as low as 0.5 ng/ml in medium significantly inhibited LPL activity (original publication **III**, Figure 5a and 5b). Exogenous recombinant Angptl4 proved to be less effective in reducing LPL activity (original publication **III**, Figure 5c), suggesting that the expression of Angptl4 and LPL within the same cell results in more efficient control of fatty acid uptake. Interestingly, LPL activity was also significantly inhibited intracellularly possibly due to an interaction between Angptl4 and LPL along the secretory pathway (original publication **III,** Figure 4c). Since GPIHBP1 was suggested to prevent the LPL inhibition by Angptl4 (Sonnenburg et al, 2009), the early interaction between the two could provide a mechanism relevant also *in vivo*, in skeletal muscle. Indeed, administration of GW501516 to mice fed a high-fat diet markedly reduced the lipid accumulation in skeletal muscle (Tanaka et al, 2003). Although increased oxidation of fatty acids is likely to play a major role, it cannot be excluded that reduced uptake due to inhibition of LPL activity by Angptl4 also contributes to decreased lipid droplets in skeletal muscle.

To provide evidence that the inhibition of LPL activity by GW501516 treatment is dependent on Angptl4 expression, *ANGPTL4* gene was silenced using siRNA technology (original publication **III,** Figure S5). As expected, the *ANGPTL4* silencing abolished the inhibitory effect of GW501516 on LPL activity in myotubes (original publication **III,** Figure 5e).

Since PPARδ is an important regulator of fatty acid oxidation and Angptl4 is the most highly upregulated gene by PPARδ, it is tempting to speculate that Angptl4 can also be involved in PPARδ-induced fatty acid oxidation (Holst et al, 2003; Luquet et al, 2003; Muoio et al, 2002; Tanaka et al, 2003). In contrast to GW501516, Angptl4 overexpression had no effect on fatty acid oxidation as measured by the production of ^{14}C-CO_2 and ^{14}C-acid soluble metabolites in myotubes (original publication **III**, Figure 6a). Because Angptl4 was suggested to upregulate lipolytic enzymes in C2C12 myocytes, the capacity of Angptl4 to enhance the

PPARδ effect on fatty acid oxidation was also evaluated (Staiger et al, 2009). A similar induction of palmitate oxidation in control and Angptl4 overexpressing myotubes was observed after incubation with a low dose of GW501516. These data strongly suggest that the induction of fatty acid oxidation by PPARδ is independent of Angptl4.

Since fatty acids are products of triacylglycerol-rich lipoprotein (TRL) hydrolysis by LPL, it is conceivable that activation of the PPARδ-Angptl4 axis functions as a negative feedback mechanism that may serve to protect the muscle fibers from lipid overload (Figure 20). The same mechanism was recently suggested to protect the heart and macrophages from lipid toxicity in mice fed a high-fat diet (Georgiadi et al, 2010; Lichtenstein et al, 2010). Although not directly measured in these studies, this effect is most likely due to the inhibition of LPL activity by fatty acid-PPARs-Angptl4 axis.

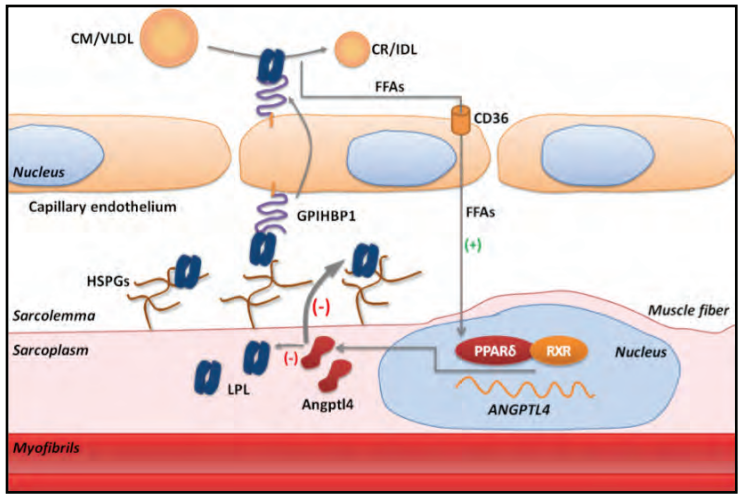

Figure 20. **Proposed negative feedback mechanism regulating LPL activity in the skeletal muscle (original publication III).** The working hypothesis is that FAs produced by LPL-mediated hydrolysis of VLDL and chylomicrons (CM) at luminal surface of the capillaries are rapidly taken up by the muscle fibers. Internalized FAs activate PPARδ which in turn upregulates *ANGPTL4* gene expression. Angptl4 inhibits LPL activity mainly at the surface of the sarcolemma where less LPL will be available to be transported at luminal site via the function of GPIHBP1. LPL inhibition by Angptl4 occurs to a lesser extent also intracellularly. This mechanism may protect the muscle from lipid overload and insulin resistance.

5.2.3 Bexarotene inhibits LPL activity via Angptl4 (unpublished data)

In order to regulate gene expression PPARs form an obligatory heterodimer with retinoic X receptor (RXR) which binds to PPAR response elements of the target genes, such as Angptl4 (Kersten, 2009; Varga et al, 2011). Bexarotene is a potent and selective RXR agonist that is currently used in the treatment of cutaneous T cell lymphoma and has promising effects in other forms of cancer or dermatologic disorders (Assaf et al, 2006; Farol & Hymes, 2004). More recently, bexarotene was shown to rapidly reduce the β-amyloid plaques and reverse cognitive deficits in mouse models of Alzheimer's disease (Cramer et al, 2012). Therefore, bexarotene is a candidate drug for the treatment of Alzheimer's disease. Unfortunately, the use of bexarotene in clinics is limited due to its undesirable increase in plasma triglycerides (Assaf et al, 2006). The mechanism underlying this side effect is not completely understood. Interestingly, a study performed in rats suggests that bexarotene-induced hypertriglyceridemia is attributable to elevated VLDL caused by inhibition of LPL activity in the muscle (Davies et al, 2001). This is further supported by the increased skeletal muscle lipoprotein lipase activity observed in RXRγ deficient mice (Haugen et al, 2004).

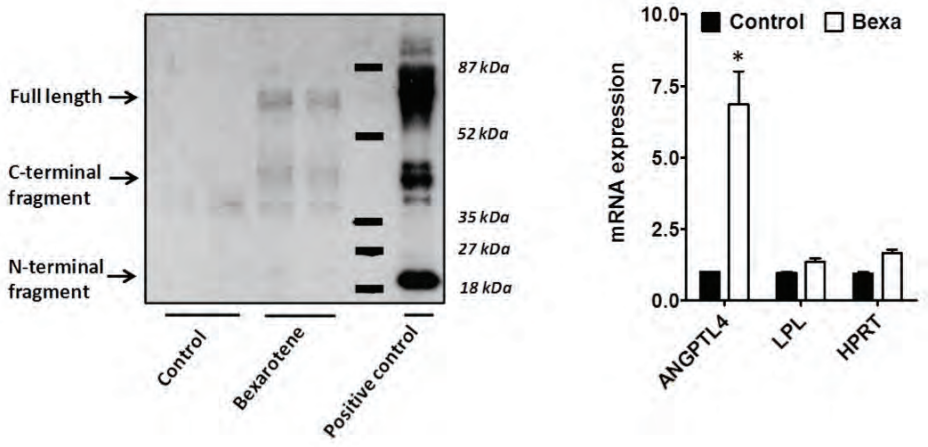

Figure 21. **Induction of Angptl4 protein and gene expression by Bexarotene in C2/LPL myotubes (unpublished data).** Secretion of Angptl4 from C2/LPL myotubes was analyzed by Western blot 48 hours after incubation with DMSO (Control) or 0.2 μM bexarotene (left panel). *ANGPTL4*, *LPL* and *HPRT* mRNA levels were measured by real time PCR in C2/LPL myotubes incubated with DMSO (Control) or 0.2 μM bexarotene for 24 hours and values are expressed relative to mouse 36B4 mRNA levels (right panel). * $p < 0.05$, t test.

To examine the involvement of Angptl4 in bexarotene-induced reduction in LPL activity, C2C12 myotubes stably overexpressing LPL (C2/LPL) were utilized (Poirier et al, 2000). Treatment of C2/LPL myotubes with bexarotene for 24 hours significantly increased Angptl4 secretion and gene expression (Figure 21). In contrast, LPL gene expression was not significantly changed after bexarotene treatment (Figure 21, right panel). In agreement with a previous report by Davies *et al.*, bexarotene significantly inhibited LPL activity in these cells (Figure 22) (Davies et al, 2001). Both silencing of *ANGPTL4* gene or inhibition of PPARδ with a specific antagonist, GSK0660, abolished the bexarotene effect on LPL activity (Figure 22). These suggest that the inhibition of LPL activity by bexarotene in skeletal muscle is dependent on Angptl4 and PPARδ.

Neutralizing antibodies targeting Angptl4 have been developed in mice and they have been shown to efficiently reduce plasma triacylglycerol levels (Desai et al, 2007). If similar antibodies could be applied to humans they might benefit patients who need bexarotene treatment. PPARδ inhibitors, such as GSK0660, can serve as an alternative treatment but their use might be limited due to the important role of PPARδ in fatty acid metabolism (Holst et al, 2003; Luquet et al, 2003; Muoio et al, 2002; Tanaka et al, 2003).

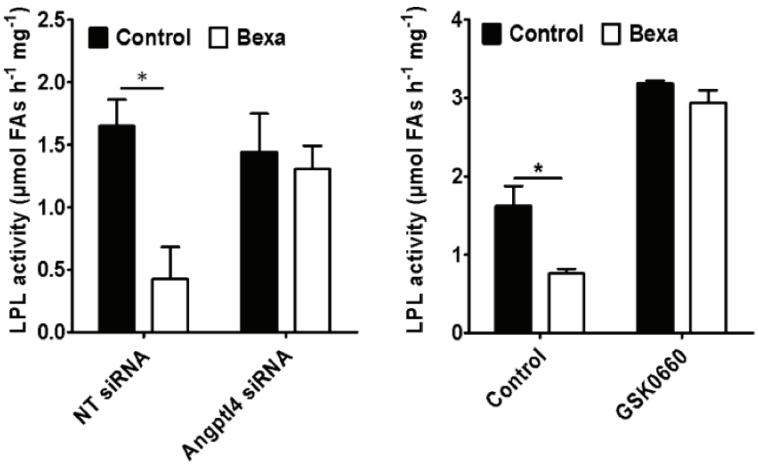

Figure 22. **Inhibition of LPL activity in C2/LPL myotubes is dependent on Angptl4 expression and PPARδ activity (unpublished data)**. C2/LPL myotubes transfected with non-targeting siRNA (NT-siRNA) or *ANGPTL4* siRNA were incubated with 0.2 µM bexarotene for 4 hours (left panel). C2/LPL myotubes pre-treated with PPARδ antagonist, GSK0660, were incubated with 0.2 µM bexarotene for 4 hours (right panel). Heparin releasable LPL activity was quantified and normalised to the protein content. * $p < 0.05$, t test**.**

5.2.4 Angptl4 and human obesity

Studies in mice demonstrated that Angptl4 not only inhibits the uptake of fatty acids by inhibiting LPL activity (extracellular lipolysis) but also increases the release of fatty acids from adipose tissue (intracellular lipolysis) (Gray et al, 2012; Mandard et al, 2006; Sanderson et al, 2009). The first observation that Angptl4 might be relevant also for human obesity came from studies in a random subsample of the Health 2000 Health Examination Survey (original publication **I**). Partial correlation analysis revealed that serum Angptl4 is inversely correlated with body mass index (BMI) ($p = 0.008$). The inverse relationship between the two parameters was evident only in subjects in the age range 30 - 45 years where the levels of Angptl4 were significantly decreased ($p = 0.03$) in overweight subjects as compared to normal weight subjects (original publication **IV**, Figure 3). Because Angptl4 is increased by endurance exercise this result could be explained by the difference in physical activity between the groups (Kersten et al, 2009). Pearson Chi-Square analysis showed that physical activity was not significantly different between the two groups ($p = 0.516$).

These results were further confirmed in monozygotic twins discordant for BMI selected from two population-based longitudinal studies, FinnTwin16 and FinnTwin12 (Kaprio, 2006). Twins with an intra-pair difference in BMI greater than 2.5 kg/m^2 were considered as discordant and those with an intra pair difference in BMI less than 2.5 kg/m^2 were considered as concordant. Serum Angptl4 levels were significantly lower in heavier twins compared with leaner co-twins ($p=0.04$) whereas no differences were observed in concordant monozygotic twins (original publication **IV**, Figure 2). Furthermore, Pearson correlations for the intra-pair differences, that control for genetic influences, showed that serum Angptl4 levels are negatively correlated with BMI ($r = -0.27$, $p = 0.003$).

Further knowledge about the relationship between Angptl4 and obesity in humans was obtained by analysing the adipose tissue *ANGPTL4* mRNA expression. *ANGPTL4* expression levels in adipose tissue were obtained from genome-wide transcriptome analysis performed in fat biopsies obtained from 44 individual monozygotic twins. In the discordant pairs, heavier twins exhibited significantly lower *ANGPTL4* expression levels as compared to their leaner co-twins whereas similar expression levels were observed in concordant twins (original publication **IV**, Figure 4). Moreover, Pearson correlation analyses revealed that *ANGPTL4* mRNA levels in adipose tissue are negatively correlated with BMI ($r=-0.44$, $p=0.001$) (original publication **IV**, Figure 5). In multivariate linear regression analyses including BMI, free fatty acids, LDL-C, HDL-C and fasting plasma glucose, only BMI significantly explained the variation in at-*ANGPTL4* (original publication **IV**, Table 2). Indications that Angptl4 could modulate adipose tissue lipolysis also in humans were obtained by analysing the expression levels of several key players involved in this

process (Lass et al, 2011). Analysis of the available data on the Affymetrix chips showed that *ANGPTL4* expression levels are positively correlated with the mRNA expression levels of *LIPE* and *ABHD5/CGI-58* (all $p<0.01$) but not with *MGLL* or *G0S2*, a recently discovered ATGL inhibitor (Yang et al, 2010) and (original publication **IV**, Figure 5). Although these results suggest a role for Angptl4 in intracellular lipolysis in humans, definite conclusions cannot be drawn based on correlation studies and gene expression levels since correlation does not mean causality and lipolysis is mainly regulated at the post-transcriptional level (Lass et al, 2011). SGBS adipocyte model was employed to directly asses the implication of Angptl4 in the human adipose tissue lipolysis (Fischer-Posovszky et al, 2008). The human Simpson-Golabi-Behmel syndrome (SGBS) preadipocytes (Figure 23, left panel) are efficiently differentiated in the presence of PPARγ agonists and in the absence of serum and albumin (Wabitsch et al, 2001). SGBS adipocytes (Figure 23, right panel) are a validated model to study the regulation of human adipose tissue lipolysis (Fischer-Posovszky et al, 2008). Incubation of SGBS adipocytes with recombinant human Angptl4 generated a two fold increase in the glycerol release, standard to evaluate intracellular lipolysis (Figure 24). In accordance to this, plasma Angptl4 was positively correlated with plasma free fatty acid levels during oral glucose tolerance test, an estimate of white adipose lipolysis (Staiger et al, 2009).

Altogether these data strongly support a role for Angptl4 in body fat regulation in humans, possibly by stimulating intracellular lipolysis in adipocytes.

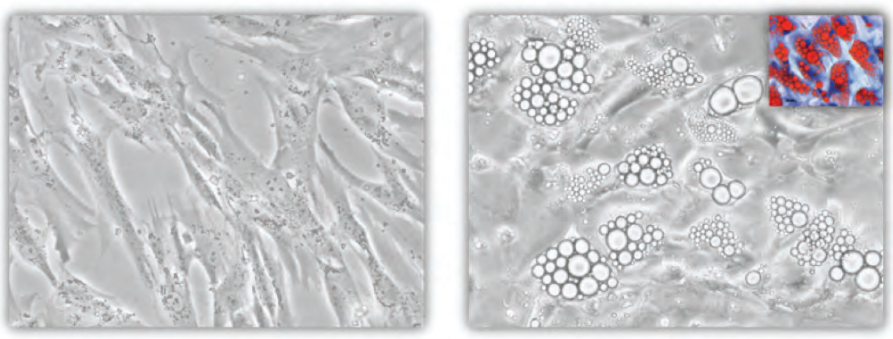

Figure 23. **Adipogenic differentiation of Simpson-Golabi-Behmel syndrome (SGBS) cells (unpublished data).** Conventional light microscopy images of SGBS preadipocytes (left panel) and SGBS adipocytes (right panel) after 14 days of differentiation**.** Inset represents SGBS adipocytes stained with haematoxylin (blue) and Oil Red O (neutral lipid staining)**.**

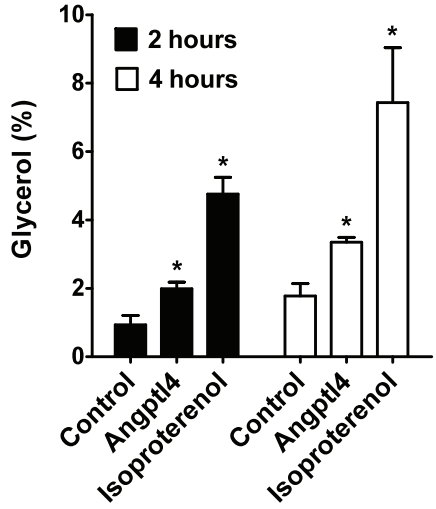

Figure 24. **The effect of Angptl4 on intracellular lipolysis in SGBS adipocytes (unpublished data).** SGBS adipocytes were incubated with 1 µg/ml recombinant human Angptl4, 100nM isoproterenol (positive control) or left untreated (control)**.** Glycerol release in the medium was quantified at 2 and 4 hours of incubation. Values are expressed as percentage of secreted glycerol from the total intracellular triacylglycerol concentration and normalised to the protein concentration. * $p < 0.05$, t test**.**

5.2.5 The effect of insulin on Angptl4 tissue expression

Insulin is a master regulator of carbohydrate and fat metabolism and impaired insulin function or lack of insulin causes diabetes. There are at least three different mechanisms by which insulin regulate triacylglycerol metabolism: (i) the secretion and uptake of TRLs by the liver (Taskinen, 2003); (ii) LPL mediated extracellular lipolysis of TRLs in adipose tissue and muscle (Wang & Eckel, 2009); (iii) inhibition of intracellular lipolysis and release of fatty acids from adipose tissue (Lass et al, 2011). Interestingly extracellular and intracellular lipolysis are also modulated by Angptl4 (Kersten, 2009). Furthermore, insulin strongly downregulates *ANGPTL4* mRNA expression in both mouse 3T3 L1 adipocytes and in adipose tissue of young healthy subjects (Ruge et al, 2011; Yamada et al, 2006). These data suggest that Angptl4 could mediate some of the insulin effects on triacylglycerol metabolism.

Skeletal muscle and liver cells in culture, as well as human subjects were studied to further explore tissue specific regulation of Angptl4 by insulin. Incubation of human myotubes, obtained from 6 nondiabetic men, in the presence of insulin for 24 hours induced a significant increase in cell associated and secreted Angptl4, as compared to non-treated cells (original publication **III**, Figure 2). This opposite effect in

comparison to adipocytes might be related to tissue specific regulation of LPL by insulin. A six hour insulin clamp decreased LPL activity in skeletal muscle while in adipose tissue LPL activity was increased (Farese et al, 1991). Skeletal muscle LPL activity seems to be less sensitive to insulin since four hour insulin clamp had no effect on LPL activity in skeletal muscle but significantly increased it in the adipose tissue (Ruge et al, 2011). Moreover, it is known that skeletal muscle LPL activity is increased during fasting and exercise, both conditions that are likely to increase also Angptl4 in skeletal muscle (Kersten et al, 2009; Ruge et al, 2005; Seip & Semenkovich, 1998). It remains to be seen if Angptl4 is a physiological regulator of LPL activity in skeletal muscle. An inhibition of the triglyceride uptake in the skeletal muscle, that is dependent on LPL activity, was observed in mice fasted for 24 hours but not in fed mice (Lichtenstein et al, 2007).

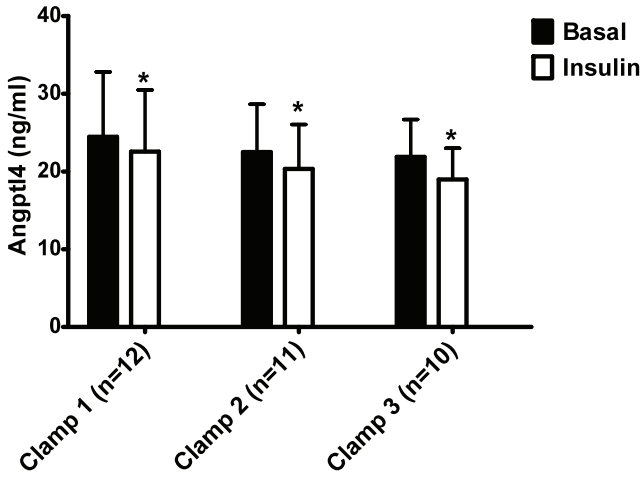

Figure 25. **Angptl4 levels before and after insulin administration (unpublished data)**. Angptl4 serum levels were measured by ELISA in serum from young healthy subjects before (Basal) and after a 4 hours hyperinsulinemic euglycemic clamp (Insulin). Results are presented as mean ± SEM of three different clamp experiments. * $p < 0.05$, Wilcoxon matched pairs test.

In contrast to myotubes and adipocytes, Angptl4 secretion in hepatocytes was not changed after insulin stimulation although some differences between the cell lines used have been observed (original publication **III**, Figure S2d). Furthermore, there is a small, but consistent, decrease in plasma Angptl4 levels after a four hour insulin clamp (Figure 25) and (Ruge et al, 2011). The small reduction in plasma Angptl4 concentration probably reflects the reduction in Angptl4 secretion from adipose

tissue, although the contribution of different organs to plasma pool is not known (Kersten, 2009).

6 CONCLUSIONS AND FUTURE PROSPECTS

It is estimated that by 2050 more than 50% of adults could be clinically obese in United Kingdom and United States (King, 2011). The trends for obesity and diabetes are expected to increase the prevalence of cardiovascular disease by approximately 10% over the next 20 years given no changes to prevention and treatment trends (Heidenreich et al, 2011). These predictions are likely to be similar in other developed countries but also in developing countries. Therefore, there is an urgent need for better implementation of prevention programs and for development of new therapies. Technological breakthroughs in human genetics achieved in the past decade indentified and validated new therapeutic targets but the understanding of their mechanisms of action is necessary for developing successful new therapies for cardiovascular disease. Such possible targets are Angptl3 and Angptl4, and this thesis provides insights into their mechanism of regulating triacylglycerol metabolism in humans.

Angptl3 showed striking differences in regulating plasma lipids. Work in this thesis suggests that HDL cholesterol is increased by Angptl3 in a concentration dependent manner whereas only complete absence of Angptl3 generated a robust decrease in plasma triacylglycerol and an increase in LPL activity. The regulation of LPL activity by Angptl3 appears to be atypical and studying the role of posttranscriptional modifications of Angptl3 such as proteolytic cleavage or glycosylation, should provide a deeper understanding of the mechanism. Very little is known about how Angptl3 regulate VLDL secretion from liver and release of free fatty acids from adipose tissue. Better characterization of its cellular distribution and interaction partners will help to understand the complex function of Angptl3 and provide a base for the development of future therapies for cardiovascular disease.

The tissue specific regulation of LPL activity by Angptl4 also deserves further investigation. Cell biology and population studies described in this thesis suggest that, in humans, Angptl4 does not act as an endocrine regulator of plasma triacylglycerol, but can have subtle roles at the tissue level. In vivo studies using animal models are the next step for better understanding the tissue specificity of this regulation. A limitation to be considered is that, in contrast to humans, high levels of Angptl4 in mouse plasma are associated with an increase in plasma triacylglycerol levels.

Data presented in this thesis clearly indicate Angptl4 as an important regulator of adipose tissue lipolysis. For a better understanding of this function, it is important to study the interacting partners of Angptl4 at the surface of adipocytes, such as

integrins and heparin sulfate proteoglycans. Furthermore, insulin is the main inhibitor of adipose tissue lipolysis and downregulates Angptl4 expression in adipose tissue. It is therefore important to study the role of Angptl4 in the insulin effect on adipose tissue lipolysis.

Angptl3 and Angptl4 have been shown to inhibit LPL-dependent extracellular lipolysis and also to increase intracellular adipose lipolysis suggesting that these two processes are closely related. It is important to clearly define whether these effects are independent or the increase in adipose tissue lipolysis is secondary to inhibition of LPL activity. This can be achieved by studying which region of the proteins is responsible for these effects since the N-terminal fragment inhibits LPL activity and C-terminal fragment might function as a signalling molecule.

Because Angptl3 and Angptl4 are secreted proteins that can have a role in the crosstalk between different organs, it is important to better understand how the different isoforms of these proteins are transported in plasma. Lipoproteins are potential transporting platforms for Angptl3 and Angptl4 and might play an important role in their function at tissue level. Moreover, it will be interesting to know how dynamic is the interaction between Angptls and lipoproteins in fed and fasted state or in dyslipidemia. ELISA methods as those described in this thesis provide the means for such studies.

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